(54) Title: SYNTHETIC POLYPEPTIDES AND ANTIBODIES RELATED TO EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN
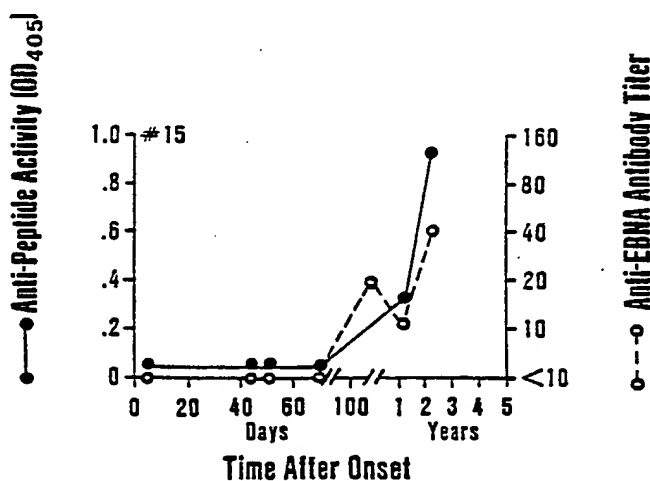
(57) Abstract

Antigens, immunogens, inocula, antibodies, diagnostic methods and systems relating to Epstein-Barr virus nuclear antigen (EBNA). Each of the compounds, compositions, methods or systems contains a synthetic, random copolymer polypeptide having about 6 to about 40 residues, or an antibody containing site that immunoreacts with such a polypeptide. The polypeptide includes the five amino acid residue sequence -Gly-R^1-Gly-R^2-Gly-, wherein R^1 and R^2 are the same or different amino acid residues selected from the group consisting of Ala, Asn, Arg, Gly, Leu, Pro, Ser, and Thr, with the provision that R^1 and R^2 are not both Gly. The polypeptide contains at least 25 mole percent Gly residues, and when linked to a carrier and introduced in an effective amount into a mammalian host is capable of inducing production of antibodies that immunoreact with EBNA.

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SYNTHETIC POLYPEPTIDES AND ANTIBODIES RELATED TO
EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN

Description

Technical Field

5   The present invention relates to immunogens,
antigens, inocula, antibodies, methods and systems
useful in the treatment and diagnosis of diseases
involving Epstein-Barr virus, and its nuclear antigen.

Background of the Invention

10   The Epstein-Barr virus (EBV) is a member of
the herpes virus family and is the causitive agent of
infectious mononucleosis (IM) in humans. EBV has
also been implicated in the pathogenesis of Burkitt's
lymphoma, nasopharyngeal carcinoma, and B lymphocyte

15   neoplasms arising in immunosuppressed patients.
Circumstantial evidence has also indicated a possible
role for this virus in human autoimmune disease such
as rheumatoid arthritis and Sjogren's Syndrome.
         EBV is an extremely common environmental

20   agent infecting 80-100 percent of the individuals
around the world. The initial or primary infection
may be acute or sub-clinical. This is followed by a
long period during which the EBV infection is latent
in B lymphocytes present in the circulating blood,

25   lymph nodes, and spleen.
         Latency is the process by which a virus is
present intracellularly in an unexpressed or
partially expressed state. This latency can be
reactivated. Although the host factors that control

30   latency in vivo are poorly known, there is some
evidence to suggest that failure of one or more
immune mechanisms is an important factor.
         Cytotoxic and suppressive T cell elements of
the immune response to EBV are reported to be very

35   important in suppressing acute infection by EBV in

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IM. They are also important in prohibiting the
uncontrolled outgrowth of B lymphocytes latently
infected with EBV.

5   Failure of T cell suppressor mechanisms is
thought to be important in allowing the emergence of
African Burkitt lymphoma, nasopharyngeal carcinoma, B
cell lymphomas arising as a consequence of
immunosuppressive therapy used to prevent rejection
of organ transplantation, and lymphomas as arising
10   during treatments of various auto immune disorders.
Epstein and Achong eds., "The Epstein-Barr Virus.",
Spring-Verleg, Berlin, Heidelberg (1970); and
Crawford et al., Lancet, 1355 (1980). In addition,
the failure of these T cell mechanisms and consequent
15   overgrowth of EBV-infected lymphocytes is thought to
play a role in reneumatoid arthritis. Slaughter et
al., J. Exp. Med., 148:1429 (1978); Depper et al., J.
immunology, 127:1899 (1981) and Tosato et al., N.
Engl. J. Med. 305:1238 (1981).
20   The serological and cell-mediated immune
responses that follow primary infection by EBV are
well documented and reflect the host's response to
the viral antigens expressed during the course of
infection. The profile of these responses as well as
25   the detection of the antigens in tissues are becoming
increasingly useful in the diagnosis of
EBV-associated diseases.
The earliest EBV-associated antigen that can
be detected after infection is EBV-induced nuclear
30   antigen (EBNA). EBNA has been detected in the
nucleus of latently-infected growth-transformed B
lymphocytes. EBNA has also been detected in the
nuclei of African Burkitt tumor lymphoblasts and
anaplastic nasopharyngeal carcinoma cells.

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The concentration of EBNA in cell nuclei of
EBV-infected By lymphocytes fluctuates during various
phases of the cell's reproductive cycle. Thus, it is
believed EBNA is cyclically being synthesized and
5   degraded. As a result of such degradation, protein
fragments (polypeptides) of EBNA traverse the
cellular cytoplasm and are believed to exist or be
expressed on the outer membrane. However, specific
EBNA degradation polypeptides have not been
10   identified to date.

It is believed that while in or on the outer
cell membrane, EBNA degradation polypeptides
constitute a significant stimulus to the host's T
lymphocytes and initiate the immune response that
15   results in the production of anti-EBNA antibodies.
It is also believed that the specific T cell response
to B cells expressing EBNA degradation polypeptides
on their surfaces may contribute to the generation of
cytotoxic and suppressive T cells important in
20   restricting growth of EBNA-containing (EBV-infected)
B lymphocytes.

Thus, assays for the presence of both EBNA
and anti-EBNA antibodies are of importance in several
common clinical situations. In addition, a vaccine
25   against EBV-infected B lymphocytes would also be of
clinical importance.

Anti-EBNA antibodies are typically assayed
using the tedious anti-complement immunofluorescence
technique (ACIF). Reedman et al., Int. J. Cancer,
30   11:499-520 (1973). This assay involves fixing
EBV-transformed human B cells to a microscope slide.
Various dilutions of a patient's serum are then added
to the fixed cells. Because anticomplementary sera
may yield false-negative reactions or prozones when
35   they are mixed with the complement (a two-stage

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procedure), it is essential to charge the test cell
smears consecutively with serum, complement, and the
anticomplement-fluorescence conjugate (a three stage
procedure).

5   There are several problems with this assay.
These include the fact that the assay is relatively
insensitive and requires amplification mediated
through complement. In addition, this assay is not
entirely specific and may not be interpreted in

10   patients whose serum contains antibodies to mammalian
cell nuclei. Still further, quantitative results
obtained using an anti-complement immunofluorescence
assay are difficult to reproduce. As a consequence
of these and other reasons, assays for anti-EBNA

15   antibodies have generally been confined to a few,
specialized laboratories.

        The above difficulties in assaying for
anti-EBNA antibodies stem from the lack of relatively
pure EBNA. Purification of EBNA from mammalian cell

20   tissue cultures is complex because of the antigen's
low concentration and polymorphology. Although it is
easier and less costly to use whole cells expressing
EBNA, as in the current technique, the problems of
specificity and reproducibility are directly the

25   result of using whole cells.

        Genetic engineering and synthetic
polypeptide technologies have recently provided
solutions to the problem of manufacturing large
quantities of protein and polypeptide antigens.

30   However, both techniques are effective only if the
amino acid residue sequence of the native protein is
known.

        The amino acid residue sequence of a natural
protein can be determined from the protein itself,

35   but this is often difficult. The gene nucleotide

sequence that codes for the protein may also reveal
the protein's amino acid residue sequence. However,
all DNA sequences have three possible reading frames,
each of which yields a different protein. Therefore,
5   the correct reading frame must be known to deduce the
correct amino acid residue sequence of a protein from
its gene.

. The correct reading frame of a DNA sequence
coding for a protein, and therefore the protein's
10   amino acid residue sequence, may be determined
through the use of antibodies. This strategy
involves manufacturing an array of protein fragments
or polypeptides whose amino acid residue sequences
correspond to the sequences obtained from the three
15   possible gene products. The protein fragments or
polypeptides that induce antibodies that immunoreact
with the gene's natural protein product thereby
indentify the gene's correct reading frame.
Conversely, if antibodies to the natural protein
20   recognize the manufactured protein fragments or
polypeptides, the relationship between gene and
protein is also established.

Heller, et al., _J. Virol._, 44:311-320
(1982), reported the DNA sequence for a portion of
25   the EBV genome that was found to contain an internal
region, designated IR3, consisting of direct repeats
of a hexanucleotide and two nonanucleotide
sequences. They cited evidence suggesting that the
sequence surrounding and including IR3 contains the
30   gene coding for EBNA. However, since it was not
known which of the three possible DNA sequence
reading frames was translated, Heller, et al., supra,
were not able definitely to deduce the amino acid
sequence for the possible EBNA protein.

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In September 1983, Hennessy and Kieff, <u>Proc.</u>
<u>Natl. Acad. Sci., U.S.A.</u>, 80:5665-5669 (1983),
reported establishing the natural reading frame for
the EBV DNA sequence reported by Heller, et al.,
5   supra. Essentially, they isolated IR3 DNA, cleaved
it into small random pieces and inserted the pieces
into the lacZ gene of an <u>E. coli</u> expression vector
such that all three EBNA gene reading frames were
expressed, each in a different clone. The lacZ gene
10   codes for beta-galactosidase, a bacterial enzyme.
The IR3-lacZ gene fusion product is expressed in <u>E.</u>
<u>Coli</u> as a fusion protein with the IR3 protein
sequence inserted between amino acids 7 and 9 (8
being deleted in the construction process) of the
15   5-galactosidase protein molecule.

Hennessy and Kieff, identified an IR3-lacZ
gene fusion that was expressing IR3 DNA in its
natural reading frame by screening for fusion
proteins that were recognized by anti-EBNA positive
20   human sera. A plasmid so identified was designated
pKH182-44.

To confirm that the protein expressed by
pKH182-44 contained EBNA-specific antigenic
determinants, Hennessy and Kieff, supra, raised
25   antisera in rabbits against cyanogen bromide-cleaved
(CNBr) IR3-galactosidase fusion protein. The CNBr
fragment used an immunogen containing 53 amino acids
homologous to EBNA and 89 amino acids homologous to
beta-galactosidase. These antisera recognized
30   natural EBNA in EBV-infected cells using indirect
immunofluorescence.

The results of Hennessy and Kieff appear to
be dependent on the repetitive nature of the EBNA IR3
domain. The fusion protein produced by pkH182-44
35   contains a relatively long segment homologous with

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the IR3 domain (e.g. 53 amino acids). It is,
therefore, not surprising that the fusion protein and
CNBr fragment thereof contained antigenic
determinants. Furthermore, Hennessy and Kieff did
5   not identify which of the sequences repeated in their
fragment were acting as antigenic determinants.

     Although Hennessy and Keiff were able to
genetically manufacture a material recognized by
anti-EBNA antibodies in human serum, it would be
10   cumbersome to use in a clinical setting because of
its design. The 53 amino acid residue segment of
their fusion protein that is homologous to EBNA is
physically and chemically part of the
beta-galactosidase protein. Its immununological
15   properties are, therefore, influenced by those
portions of the beta-galactosidase molecule from
which it cannot be separated. In fact, all of the
human sera used in their study reacted with
beta-galactosidase, and required treatment with
20   beta-galactosidase to adsorb and remove this
reactivity before testing for specificity against the
genetically manufactured protein.

     Another approach to the interrelated
problems of determining a gene's correct reading
25   frame and manufacturing large quantities of
pathogen-related antigens (immunogens) for clinical
and diagnostic purposes is the use of synthetic
polypeptide chemistry. This method of manufacturing
antigens (immunogens) has an advantage over the
30   genetic engineering methods described above.
Synthetic polypeptide antigens do not contain natural
protein by-products or fragments thereof, and thereby
their use eliminates the possibility of unwanted
cross reactivity and the need to pretreat serum
35   samples as in the Hennessy and Kieff study.

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While the general concept of preparing
synthetic antigens (immunogens) and using them to
induce antibodies of predetermined specificity has
been described, there remains a large area of this
5   technology that continues to defy predictability.
There are at least two reasons for this. First, a
synthetic antigen (immunogen) does not necessarily
induce antibodies that immunoreact with the intact
protein in its native environment. Second, a host's
10   natural antibodies to a naturally occurring immunogen
such as a viral protein rarely immunoreact with a
polypeptide that corresponds to a short linear
portion of the immunogen's amino acid residue
sequence. This latter phenomenon is believed to be
15   the result of short linear polypeptides lacking
required secondary and tertiary conformational
structures.

Much of the work on the binding of peptide
by antibody made to proteins is summarized in a
20   review by Benjamini, E., et al., _Current Topics in
Microbiology and Immunology_ 58:85-134 (1972). The
role of peptide structure in antibody binding has
been emphasized by Goodman, J. W., _Immunochem_
6:139-149 (1969).

25   Most of the studies concerned with how
changes in the sequence of peptides effect antibody
binding have been interpreted as indicating that the
structure of the antibody combining site plays a
predominant role. The effect of sequence and
30   structural changes in these studies is intermixed and
difficult to segregate. Some of these studies can
equally well be explained by structural changes in
antigen effecting the binding.

Antibody response at the molecular level
35   involves binding of an antigen of defined sequence

(primary structure) and in a defined conformation
(secondary and tertiary structure). Immune response
to protein antigens has traditionally been
interpreted as being directed against primary,

5   secondary or tertiary structue of the protein.

This classification scheme may have some
validity for proteins that have a well defined
overall structure at physiological temperatures and
solutions. However, its validity is in doubt for

10 peptide antigens that have a more dynamic structure.

Several groups have reported structural
studies of polymers of repeating sequence of glycine
and alanine or glycine and serine that were
synthesized as models of silk fibroins [Anderson et

15 al., J. Mol. Biol. 67:459-468 (1972)] and collagen
[Anderson et al., BBRC 39:802-808 (1970); Doyle et
al., J. Mol. Biol. 51:47-59 (1970]. The most
systematic study has been that of Brack et al.,
Biopolymers 11:563-586 (1972) who reported synthesis

20 of a series of block homopolymeric polypeptides in
which the homopolymeric block repeating units had the
formula $(Ala_x$-$Gly_y)$ wherein when $x = 1$,
$y = 1, 2$ and 3; when $x = 2$, $y = 1, 2$ and 3; and when
$x = 3$, $y = 3$.

25 The results reported from this latter study
were that in the solid state the block homopolymers
composed mostly of alanine were alpha-helical, and
those containing mostly glycine were disordered. In
solution, polyalanine was reported to be

30 alpha-helical, but poly-$(Ala_2$-$Gly_1)$ was reported
to be in beta-antiparallel form. The more
glycine-rich polymers were said to have another fixed
structure that was reported as neither alpha-helix
nor the beta-structure.

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The homopolymerized blocks of glycine and
alanine reported by Brack et al. were prepared by the
condensation polymerization of the di- through
hexapeptide repeating units having a

5   carboxyl-terminal glycyl residue in active ester
form. Degrees of polymerization from 2 through 68
were reported for poly(Ala-Gly$_2$).

Even though the solvents used in those
studies were not physiologically acceptable, e.g.,

10   water or phosphate buffered saline, the results
illustrate two points: (1) structural changes can
occur as the sequence of a polypeptide changes, and
(2) structural changes also occur during the
transition from solution to solid state.

15   Summary of Invention

The present invention contemplates a
synthetic, random copolymeric polypeptide capable of
inducing the production of antibodies that
immunoreact with Epstein-Barr Virus Nuclear Antigen

20   (EBNA). The random copolymeric polypeptide contains
about 6 to about 40, and preferably about 15 to about
20, amino acid residues, and includes the amino acid
residue sequence, written from left to right and in
the direction of amino-terminus to carboxy-terminus,

25   represented by the formula,

        -Gly-R^1-Gly-R^2-Gly-

        wherein:
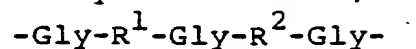
        R^1 and R^2 designate amino acid residues
which taken individually are the same or different

30   and are selected from the group consisting of Ala,
Asn, Arg, Gly, Leu, Pro, Ser and Thr, with the
provision that R^1 and R^2 cannot both be Gly. The
polypeptide contains at least 25 mole percent Gly
residues, and is capable, when linked to a carrier

35   and introduced in an effective amount into a

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mammalian host, of inducing production of antibodies
that immunoreact with EBNA.

Preferred polypeptides of this invention
include an amino acid residue sequence selected from
5   the group consisting of:

(i)   -Gly-Arg-Gly-Arg-Gly-;

(ii)   -Gly-Asn-Gly-Leu-Gly-; and

(iii)   -Gly-Ser-Gly-Ser-Gly-.

In one more preferred embodiment, R^1 is
10 Ala and R^2 is Ala, so that the polypeptide contains
the sequence -Gly-Ala-Gly-Ala-Gly-.

In another more preferred embodiment, R^1
is Ala and R^2 is Gly, so that the polypeptide
contains the sequence -Gly-Ala-Gly-Gly-Gly-.

15 In still another more preferred embodiment,
R^1 is Gly and R^2 is Ala, so that the polypeptide
contains the sequence -Gly-Gly-Gly-Ala-Gly-.

A preferred amino acid residue sequence
includes a sequence represented by the formulae,
20 taken from left to right and in the direction of
amino-terminus to carboxy-terminus, selected from the
group consisting of:

-Arg-Ala-Arg-Gly-Arg-Gly-Arg-Gly-Arg-Gly-Glu-
Lys-Arg-Pro-Met-;

25   -Ile-Met-Ser-Asp-Glu-Gly-Pro-Gly-Thr-Gly-Asn-
Gly-Leu-Gly-Glu-;

-Pro-Gly-Ala-Pro-Gly-Gly-Ser-Gly-Ser-Gly-Pro-;
the pharmaceutically acceptable salts thereof, and
antigenically related variants thereof. Also
30 preferred are the corresponding polypeptides
themselves, that is,

H-Arg-Ala-Arg-Gly-Arg-Gly-Arg-Gly-Arg-Gly-Glu-
Lys-Arg-Pro-Met-OH;

H-Ile-Met-Ser-Asp-Glu-Gly-Pro-Gly-Thr-Gly-Asn-
35 Gly-Leu-Gly-Glu-OH;

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H-Pro-Gly-Ala-Pro-Gly-Gly-Ser-Gly-Ser-Gly-Pro-
OH; the pharmaceutically acceptable salts thereof,
and antigenically related variants thereof.

In a particularly preferred embodiment, the
5   synthetic, random copolymer polypeptide contains
about 8 to about 40, and preferably about 15 to about
20, amino acid residues and includes the
before-defined -Gly-R^3-Gly-R^4-Gly- amino acid
residue sequence. This polypeptide also: (a)
10   contains at least about 50 mole percent Gly residues,
(b) is capable, when linked to a carrier and
introduced in an effective amount into a mammalian
host, of inducing the production of antibodies that
immunoreact with EBNA, (c) is capable of
15   immunoreacting with human antibodies induced by
natural EBNA, and (d) additionally includes the
overlapping six amino acid residue sequence, written
from left to right and in the direction of amino-
terminus to carboxy-terminus, represented by the
20   formula,
                    -Gly-Ala-Gly-Gly-Ala-Gly-.

A particularly preferred amino acid residue
sequence includes a sequence represented by the
formulae, taken from left to right and in the
25   direction of amino-terminus to carboxy-terminus,
selected from the group consisting of:
     (i)   -Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
               Gly-Gly-Gly-Gly-Arg-;
     (ii)   -Lys-Gly-Thr-His-Gly-Gly-Thr-Gly-Ala-Gly-Ala-
30   Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-;
     (iii)   -Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-
               Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly;
     (iv)   -Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
               Gly-Gly-Gly-Ala-Gly-;

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(v)   -Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-
         Gly-Gly-Ala-Gly-Gly-;

(vi)   -Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
         Gly-Gly-Ala-Gly-Gly-Gly-Ala-Gly-
         Ala-Gly-;

(vii)   -Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-
         Gly-Gly-Ala-Gly-Ala-Gly-;

(viii)   -Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-
         Ala-Gly-Ala-Gly-;

the pharmaceutically acceptable salts thereof, and
antigenically related variants thereof. Also more
preferred are the corresponding polypetides
themselves, that is,

(i)   H-Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
         Gly-Gly-Gly-Gly-Arg-OH;

(ii)   H-Lys-Gly-Thr-His-Gly-Gly-Thr-Gly-Ala-Gly-Ala-
         Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-OH;

(iii)   H-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
         Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-OH;

(iv)   H-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
         Gly-Gly-Ala-Gly-OH;

(v)   H-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-
         Gly-Gly-Ala-Gly-Gly-OH;

(vi)   H-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
         Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
         Gly-OH;

(vii)   H-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-
         Gly-Gly-Ala-Gly-Ala-Gly-OH;

(viii)   H-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-
         Ala-Gly-OH;

the pharmaceutically acceptable salts thereof and
antigencally related variants thereof.

          The present invention also contemplates a
synthetic multimer containing a plurality of joined
synthetic polypeptide repeating units wherein at

least one of the repeating units is a polypeptide as described above. The polypeptide repeating units may be joined in a head-to-tail manner by amide bonds. Alternatively, the synthetic polypeptide monomers may

5   be joined by other than amide bonds to form a polymeric multimer such as through the use of intramolecular, interpolypeptide cysteine disulfide bonds.

        In another embodiment, an effective amount

10   of a polypeptide of this invention is used in a physiologically tolerable diluent to form an inoculum capable of inducing antibodies that immunoreact with EBNA. In addition to being used for the production of antibodies, an inoculum of this invention may be

15   used as a vaccine in humans as a means for inducing active immunity to lymphocytes expressing EBNA or fragments thereof on their cell surface.

        In still another embodiment, a receptor molecule is contemplated that contains an antibody

20   combining site that is capable of immunoreacting with EBNA. The receptor is raised to a synthetic immunogen comprising a synthetic polypeptide described above alone or as a conjugate.

        Also contemplated is a diagnostic system for

25   assaying for the presence of EBNA. The system comprises receptor molecules as described above and an indicating means for signaling for the immunoreaction of the combining sites with EBNA.

        Further contemplated is a diagnostic system

30   for assaying for the presence of antibody molecules to EBNA in a body component. Such a system comprises a particularly preferred, random copolymer synthetic polypeptide as described above and an indicator means for signaling the immunoreaction of the polypeptide

35   with the antibody molecules to EBNA. In a more

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preferred embodiment, this system also contains a
solid support comprised of a solid matrix to which
the particularly preferred polypeptide is affixed. A
means for identifying the isotype of the

5   immunoreacted antibody molecules may also be included
in the system.

         Additionally contemplated is a preparation
for passive immunization against B lymphocytes
expressing EBNA on their cell surfaces. The

10   preparation comprises an effective amount of a
receptor molecule described above in a
physiologically tolerable diluent. When introduced
into a mammalian host, the preparation is capable of
reducing the effect on the host of B lymphocytes

15   expressing EBNA on their cell surfaces.

Brief Description of the Drawings

         In the Figures forming a portion of the
disclosure of this invention:

         Figure 1 is a plot of the circular dicnroism

20   spectra of polypeptides (F), (B) and (E). These
polypeptides are also referred to herein as
polypeptides F13, F62 and F12, respectively. Each
spectrum is the average of 10 successive scans of a
polypeptide in physiological solution (phosphate

25   buffered saline) at a concentration of 1
milligram/milliliter (mg/ml). The optical rotation
is expressed as milliradians and is plotted against
the polarized light wavelength, expressed in
nanometers (nm). The relatively featureless plot for

30   polypeptide F (F13) is indicative of a random
conformation that is the usual result obtained with
peptides of this size. The trough and peaks spectrum
demonstrated by polypeptide B (P62) is characteristic
of a relatively stable secondary structure or

35   conformation, probably beta-pleat. Although the data

are not shown, the polypeptides P27, P60, F14 and F15
have very similar spectra indicating that the more
preferred polypeptides of this invention exist as
similar stable conformations in physiological

5   solution. The spectrum of polypeptide E (F12)
indicates partial assumption of such conformation.

Figure 2 is a photograph of nitrocellulose
immunoblots of whole cell extracts of EBV-
transformed WI-L2 cells using rabbit antipeptide

10   antisera to synthetic polypeptides C (P60) and B
(P62). A human serum (from patient TJ) previously
defined as anti-EBNA positive; i.e., containing
anti-EBNA antibodies, was used as a positive control
in lane A at a 1:20 dilution. Rabbit anti-P60 (C)

15   serum at a 1:50 dilution (lane B) and rabbit anti-P62
(B) serum at 1:10 (lane D) immunoreacted with the
same band as the positive control indicating they
recognize natural EBNA. Lanes A-G are identified at
the bottom of the Figure.

20   Recognition of EBNA by anti-P60 serum was
innibited by incubating anti-P60 serum diluted 1:50
with 40 micorgrams/milliliter (ug/ml) of polypeptide
P60 for one hour prior to immunoblotting (lane 3).
Similarly, recognition of EBNA by anti-P62 serum was

25   inhibited by incubating anti-P62 serum diluted 1:10
with 40 ug/ml of polypeptide P62 for one hour prior
to immunoblotting.

The antigenic relatedness of P60 and P62 is
demonstrated in lanes 6 and 7. Lane 6 shows anti-P62

30   serum diluted 1:10 immunoreacting with the EBNA
band. In lane 7, the immunoreactivity of anti-P62
serum with the EBNA band was inhibited by incubation
with polypeptide P60 at 40 ug/ml for one hour prior
to immunoblotting.

35   Figure 3 is a graph illustrating the
inhibition of anti-P62 serum activity in serum

EBNA-positive of patient 1011 by a competing
polypeptide in solution. An ELISA using polypeptide
P62 as the solid phase target was performed using
serum of patient 1011 pre-incubated for one hour with
5   either of polypeptides P27, P62, P60, P89 or F16
before use in the ELISA. Polypeptides P27, P62, P60,
P89 and P16 are also referred to herein as
polypeptides A, B, C, D, and G, respectively. The
percent anti-polypeptide activity is plotted as the
10   ordinate versus the concentration of the competing
polypeptide in micrograms/per milliliter (ug/ml).

Figure 4 is a graph that illustrates the
parallel time course of appearance of antiboides to
EBNA (dashed line, O) and polypeptide P62 (solid
15   line, ●) in a case of documented infectious
mononucleosis (IM). Sequential sera were collected
after clinical onset and were titered for anti-EBNA
activity following the procedure reported in
Catalano, et al., J. Clin. Invest., 65:1238-1242
20   (1980) on the right-hand ordinate. The serum samples
were also assayed in the ELISA of this invention
using polypeptide P62 as the solid phase target as is
shown on the left-hand ordinate wherein the optical
density at 405 nanometers (OD_{405}) is plotted.
25   Figure 5 is composed of two graphs that
illustrate the early detection of antibodies to
polypeptide P62 (solid line, ●) as compared to
anti-EBNA (dashed line, O) using detection by the
classical ACIF method described by Henle, G. et al.,
30   J. Infect. Dis., 130:231 (1974). Sequential sera
were collected from two patients (#14 top panel, #2
bottom panel) with clinically documented infectious
mononucleosis. The sera were titered for anti-EBNA
activity as reported in Catalano, et al., above.
35   Anti-polypeptide activity was measured using the

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ELISA of this invention using polypeptide P62 as the solid phase target, with the activity being reported as in Figure 4.

Detailed Description of the Invention

5   I.   Introduction

Humans infected with Epstein-Barr virus (EBV) develop antibodies against a viral nuclear antigen (EBNA) that is present in virally-transformed B lymphocytes. Traditional clinical techniques used

10   to assay for EBNA and anti-EBNA antibodies in humans are cumbersome. In addition, current procedures for the purification of EBNA from cell culture are not readily adaptable to mass production.

The present invention contemplates the use

15   of synthetic polypeptide technology· to overcome some of the problems of the current methodologies. Short synthetic polypeptides may immunologically mimic antigenic determinants on a natural protein and may therefore be used to raise antibodies of

20   predetermined specificity that recognize the natural protein.

The phrase "immunologically mimics" is used herein to mean that an immunogenic polypeptide of this invention induces production of antibodies that

25   bind to the inducing polypeptide and also to the cognate sequence in the intact protein. This phenomenon may be used both experimentally and clinically.

Experimentally, antibodies to synthetic

30   polypeptides may be used to establish the DNA reading frame, and therefore the amino acid residue sequence of a clinically important protein such as EBNA. Clinically, antibodies of predetermined specificity raised to synthetic polypeptides may be used for

35   diagnostic and therapeutic purposes.

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Heller, et. al., supra, reported a DNA
nucleotide sequence with characteristics that
indicated it might contain the gene coding for EBNA.
They predicted that if the DNA was translated into
5   protein, the three possible reading frames would code
for an IR3 protein domain of more than 200 amino acid
residues composed of only (i) serine, arginine, and
glycine; (ii) glycine and alanine; or (iii)
glutamine, glutamate, and glycine, depending upon the
10   DNA reading frame expressed.

The reported chemical properties of the EBNA
molecule, when taken together with the distribution
of possible stop codons in the EBNA gene, indicated
that the IR3 was composed primarily of glycine and
15   alanine residues.

To assess that indication, short
polypeptides were synthesized whose amino acid
residue sequences substantially correspond to that of
an EBNA protein whose IR3 is a glycine-alanine random
20   copolymer.

A.   Synthetic Polypeptides

1.   Sequences

The series of small synthetic polypeptides
(5-21 amino acid residues in length) used in this
25   study were synthesized using the solid phase method
of Merrifield. Merrifield et. al., J. Am. Chem. Soc.,
85:2149-2154 (1963). The sequences were chosen to
represent different areas from within and just
outside the proposed IR3 region of the EBNA.
30   The term "synthetic" as used herein means
that the polypeptide molecule or polypeptide
repeating unit has been built up by chemical means;
i.e., chemically synthesized, rather than being
prepared by a biological means, as by genetic
35   engineering techniques. Thus, the synthetic

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polypeptides embodying the present invention are free from naturally occurring proteins and fragments thereof.

The chemically synthesized polypeptides also
5   therefore differ from degradation products of naturally occurring proteins as are prepared by the action of cyanogen bromide on the protein. The well-known solid phase chemical synthesis in which blocked amino acid residues are added in a serial
10   manner to obtain the desired polypeptide is the preferred method of synthesis, and is discussed in greater detail hereinbelow.

All amino acid residues identified herein are in the natural or L-configuration. In keeping
15   with standard polypeptide nomenclature, abbreviations for amino acid residues are as follows:

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	L-tyrosine
G	Gly	L-glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L- proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
Z	Glx	L-glutamic acid or L-glutamine

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W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
B	Asx	L-aspartic acid or L-asparagine
C	Cys	L-cysteine

The present invention contemplates a synthetic, random copolymer polypeptide containing about 6 to about 40 amino acid residues, preferably about 15 to about 20 amino acid residues, and including the sequence defined by the formula written from left to right and in the direction of amino-terminus to carboxy-terminus

$$-Gly-R^1-Gly-R^2-Gly-$$
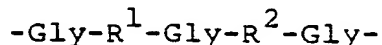
wherein R^1 and R^2 designate amino acid residues which when taken individually are the same or different and are Ala, Asn, Arg, Gly, Leu, Pro, Ser and Thr, provided that R^1 and R^2 are not both Gly. The polypeptide also contains at least 25 mole percent glycine residues, and is capable, when linked to a carrier and introduced in an effective amount into a mammalian host, of inducing production of antibodies that immunoreact with EBNA.

In one preferred embodiment, R^1 and R^2 are both Arg so that the polypeptide includes the amino acid residue sequence: -Gly-Arg-Gly-Arg-Gly-. In another preferred embodiment, R^1 is Asn and R^2 is Leu so that the polypeptide includes the amino acid residue sequence: -Gly-Asn-Gly-Leu-Gly-. In still another preferred embodiment, R^1 and R^2 are both Ser so that the polypeptide includes the amino acid residue sequence: -Gly-Ser-Gly-Ser-Gly-.

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Preferred amino acid residue sequences
include the sequences, taken from left to right and
in the direction of amino-terminus to
carboxy-terminus, represented by the formulae:

5   -Arg-Ala-Arg-Gly-Arg-Gly-Arg-Gly-Arg-Gly-Glu-
Lys-Arg-Pro-Met-;
           -Ile-Met-Ser-Asp-Glu-Gly-Pro-Gly-Thr-Gly-Asn-
Gly-Leu-Gly-Glu-
           Pro-Gly-Ala-Pro-Gly-Gly-Ser-Gly-Ser-Gly-Pro-;

10 the pharmaceutically acceptable salts thereof, and
antigenically related variants thereof.

In more preferred embodiments, R^1 and R^2
are Ala or Gly. For example, R^1 may be Ala and
R^2 may be Ala; R^1 may be Ala and R^2 may be Gly;

15 and R^1 may be Gly and R^2 may be Ala. The more
preferred embodiments thus include a five amino acid
residue sequence represented by a formula selected
from the group consisting of

           (i)   -Gly-Ala-Gly-Ala-Gly-;

20   (ii)   -Gly-Ala-Gly-Gly-Gly-; and
           (iii)   -Gly-Gly-Gly-Ala-Gly-.

The term "random copolymer" is used herein
in its usual meaning. Thus, the polypeptides are
copolymers because they contain a plurality of

25 different amino acid residue repeating units. The
copolymers are random as compared to alternating or
block copolymers because the individual amino acid
residues of the polypeptides are not present in a
particular repeating sequence as is found in the

30 repeating sequences of an alternating copolymer or
the homoblock copolymers prepared by Anderson et al.,
Doyle et al., or Brack et al., supra.

Thus, even though the polypeptide
denominated P62 (Table 1) that contains the

35 sequentially repeating sequence

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-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-, that polypeptide additionally contains an -Ala-Gly- peptide at the carboxyl-terminus. As a consequence, there is no amino acid residue sequence that repeats

5　throughout the polypeptide, and polypeptide P62 must be viewed as being a random copolymer and not a homoblock copolymer as are the $poly(Ala_x-Gly_y)$ materials prepared by Brack et al. or the poly(Ser-Gly) materials prepared by Anderson et al.

10　whose identical blocks of particular amino acid residue sequences repeat throughout the length of their polymers.

The synthetic, random copolymer polypeptides of this invention are often referred to herein simply

15　as "polypeptides" or as "synthetic polypeptides". That usage is for brevity.

The term "antigenically related variants" is used herein to designate polypeptides of differing overall amino acid residue sequence that share at

20　least a portion of one antigenic determinant and are therefore immunologically cross-reactive.

The term "antigenic determinant", as used herein, designates the structural component of a molecule that is responsible for specific interaction

25　with corresponding antibody (immunoglobulin) molecules elicited by the same or related antigen or immunogen.

The term "immunogenic determinant", as used herein, designates the structural component of a

30　molecule that is responsible for the induction in a host of an antibody containing an antibody combining site (idiotype) that binds with the immunogen when used as an antigen.

The term "antigen", as used herein, means an

35　entity that is bound by an antibody.

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The term "immunogen", as used herein,
describes an entity that induces antibody production
in the host animal. In some instances, the antigen
and immunogen are the same entity, while in other
5   instances, the two entities are different.

For example, as is described hereinafter,
polypeptide P62 was used to induce production of
antibodies in a rabbit and thus, was used as an
immunogen. The antibodies so induced bind to
10   polypeptide P62 when used as an antigen. Polypeptide
P62 was therefore both an immunogen and an antigen.
Anti-EBNA antibodies bind to both EBNA the immunogen
and antigen as well as to polypeptide P62 as antigen.

Preferred embodiments of the present
15   invention are the synthetic, random copolymer
polypeptides P89, F12, F13, as shown in Table 1
below, the pharmaceutically acceptable salts thereof,
and antigenically related variants thereof. Each of
those polypeptides contains a $-Gly-R^1-Gly-R^2-Gly-$
20   amino acid residue sequence, where R^1 and R^2 are
as before defined; each polypeptide contains at least
about 25 mole percent Gly; and each is capable of
inducing antibodies that bind to EBNA, as described
before.

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TABLE I

SEQUENCES OF SYNTHETIC POLYPEPTIDES

	Peptide*	Sequence
	P60(C)	H-Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala- Gly-Gly-Gly-Gly-Arg-OH;
5	P89(D)	H-Arg-Ala-Arg-Gly-Arg-Gly-Arg-Gly-Arg-Gly-Glu- Lys-Arg-Pro-Met-OH;
	F12(E)	H-Ile-Met-Ser-Asp-Glu-Gly-Pro-Gly-Thr-Gly-Asn- Gly-Leu-Gly-Glu-OH;
10	F13(F)	H-Pro-Gly-Ala-Pro-Gly-Gly-Ser-Gly-Ser-Gly- Pro-OH;
	P27(A)	H-Lys-Gly-Thr-His-Gly-Gly-Thr-Gly-Ala-Gly-Ala- Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-OH;
15	P62(B)	H-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala- Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala- Gly-OH;
	F14	H-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala- Gly-Gly-Gly-Ala-Gly-OH;
20	F15	H-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala- Gly-Gly-Ala-Gly-Gly-OH;
	F16(G)	H-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala- Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala- Gly-OH;

25   * Parenthesized capital letters are used to
designate the corresponding polypeptide in some of
the Figures and Tables herein.

30   The results of polypeptide/anti-polypeptide
receptor binding and binding inhibition studies are .
discussed hereinafter in section I D. Those results,
illustrate cross-reactivities and cross-inhibitory
effects that parallel the amount of sequence homology
35 among the polypeptides. For example, polypeptide

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P60 contains a 10 amino acid segment homologous with P62. Polypeptide P27 contains an 8 amino acid segment homologous to polypeptides P60, P62 and Dl (Table 2). Polypeptide D2 (Table 2) contains a seven

5   amino acid residue segment homologous to segments of polypeptides P27, P60, P62 and Dl. Polypeptide P89 which did not significantly crossreact in the study, contains no sequence homology with polypeptides P27, P62, and P60.

10   More importantly, the 8 amino acid residue sequence shared by polypeptides P27, P62, P60 and Dl contains at least one antigenic determinant common to all three random copolymer polypeptides, thereby making those three polypeptides antigenically related

15   varients. In addition, the shared segment includes the six amino acid residue sequence
                   -Gly-Ala-Gly-Gly-Ala-Gly-
     and an overlapping sequence represented by the formula
     -Gly-R^1-Gly-R^2-Gly- wherein R^1 and R^2 are

20   both Ala; i.e., -Gly-Ala-Gly-Ala-Gly-.

          By "overlapping", it is meant that the second-named sequence is contained in part of the first-named sequence. This overlapping of amino acid residue seqence is illustrated for polypeptide P62 by

25   the overlapping, "boxed", sequence portions shown below in which the single letter amino acid residue code is used.

30   G-G-G-A-G-G-A-G-A-G-G-G-A-G-G
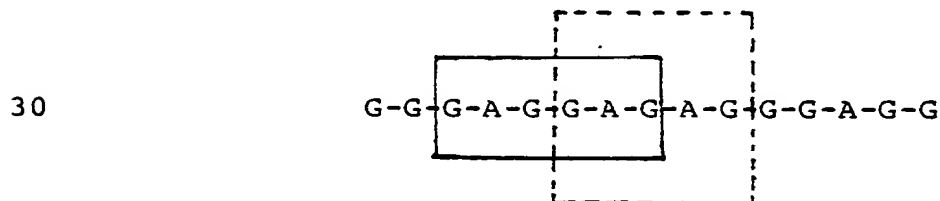
          Synthetic, random copolymer polypeptides

35   that contain both the shared six amino acid

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residues-containing sequence and the overlapping five
amino acid residue constitute a still more,
particularly, preferred embodiment of this
invention.   In such particularly preferred
5   embodiments, the synthetic, random copolymer
polypeptide of the present invention contains about 8
to about 40, and preferably about 15 to about 20,
amino acid residues and includes, in addition to the
before defined -Gly-R^1-Gly-R^2-Gly- amino acid
10   residue sequence, the sequence, written from left to
right and in the direction of amino-terminus to
carboxy-terminus, represented by the formula,

              -Gly-Ala-Gly-Gly-Ala-Gly-,

contains at least about 50 mole percent Gly residues
15   and is capable of both (a) inducing the production of
antibodies that immunoreact with EBNA when linked to
a carrier and introduced in an effective amount into
a mammalian host, and (b) immunoreacting with human
antibodies induced by natural EBNA.

20   Particularly preferred amino acid residue
sequences include the sequences, taken from left to
right and in the direction of amino-terminus to
carboxy-terminus, represented by the formulae:

(i)   -Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-
25   Gly-Ala-Gly-Gly-Gly-Gly-Arg-;

(ii)   -Lys-Gly-Thr-His-Gly-Gly-Thr-Gly-Ala-
               Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-;

(iii)   -Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-
               Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-
30   Ala-Gly-;

(iv)   -Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-
               Gly-Ala-Gly-Gly-Gly-Ala-Gly-;

(v)   -Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-
               Gly-Ala-Gly-Gly-Ala-Gly-Gly-;

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(vi)   -Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-
            Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-
            Ala-Gly-;

(vii)   -Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
            Gly-Gly-Gly-Ala-Gly-Ala-Gly-;

(viii)   -Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-
            Ala-Gly-Ala-Gly-;

pharmaceutically acceptable salts thereof, and
antigenically related variants thereof.

It is noted that a dash at the beginning or
end of an amino acid residue sequence indicates a
bond to a radical such as H and OH, at the amino- and
carboxy-termini, respectively, or a further sequence
of one or more amino acid residues up to a total of
forty amino acid residues in the polypeptide chain.

Also particularly preferred are the
corresponding polypeptides themselves, that is,
polypeptides P60, P27, P62, F14, F15, and F16 shown
in Table 1 and D1 and D2 shown in Table 2, and the
pharmaceutically acceptable salts thereof and
antigenically related variants thereof.

The phrase "pharmaceutically acceptable
salts", as used herein, refers to non-toxic alkali
metal, alkaline earth metal and ammonium salts used
in the pharmaceutical industry, including the sodium,
potassium, lithium, calcium, magnesium and ammonium
salts and the like that are prepared by methods
well-known in the art. The phrase also includes
non-toxic acid addition salts that are generally
prepared by reacting the compounds of this invention
with a suitable organic or inorganic acid.
Representative salts include the hydrochloride,
hydrobromide, sulfate, bisulfate, acetate, oxalate,
valerate, oleate, laurate, vorate, benzoate, lactate,

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phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, and the like.

2. <u>Size</u>

The effect of changing the size of the synthetic polypeptide on its ability to be bound by anti-EBNA antibodies was studied. It was generally found that as polypeptide size is decreased, its ability to be bound by antibodies also decreased.

Polypeptide P62 consists of 20 amino acid residues of which the first nine residues from the amino-terminus are directly repeated, as shown in Table 2 using the single letter code for amino acid residues. A series of polypeptides homologous to P62 was synthesized in which amino acid residue triads were deleted from the amino-terminus of the preceding peptide to provide polypeptides D1, D2 and D3 as are shown in Table 2, below. Increasing portions of the sequence symmetry of P62 are therefore missing in polypeptides D1, D2 and D3.

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TABLE 2

Polypeptide Designation	Amino Acid Residue Sequence[#]
P62:	A G A G G G A G G *A G A G G G A G G A G
D1 :	G G G A G G A G A G G G A G G A G
D2 :	A G G A G A G G G A G G A G
D3 :	A G A G G G A G G A G
A5 :	G G G A G
A6 :	A G G G A G
A7 :	G A G G G A G
A8 :	A G A G G G A G
A9 :	G A G A G G G A G

[#] Polypeptide sequences using the single letter code
are shown in the direction from left to right and
from amino-terminus to carboxy-terminus.
* Junction of 9-mer direct repeat in polypeptide P62.

The ability of the D series of polypeptides
to immunroreact with 3 human anti-EBNA sera and
rabbit anti-P62 serum was studied using the
polypeptides in the solid phase of the ELISA,
described hereinafter. Antibody binding to D1 was
nearly the same as that to the parent polypeptide P62
for all sera tested. In contrast, there was no
binding to solid phase D3 for any serum except rabbit
anti-P62. The results for D2 were intermediate and
were dependent upon the serum tested. Thus, antibody
recognition of this series of polypeptides was

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decreased as polypeptide length was shortened from 20
to 11 amino acid residues.

5   The fact that antibody binding fell off for
all the antisera argues against the possibility that
a specific antigenic determinant was being deleted by
the sequential elimination of amino acid residues
since the polypeptides contained two antigenic
determinants, only one of which was affected by the
sequence deletions. Also, the sequence symmetry of
10   P62 assured that all sequences of four to eight amino
acid residues that were present in P62 were also in
D3, with the exception of those across the juncton of
the repeat.

        Conformational changes in the polypeptides
15   bound to the solid support in the ELISA may have
contributed to the suppression of antibody
recognition. This possibility was studied by
inhibiting the binding (immunoreacting) of several
sera to solid phase-bound P62 by use of varying
20   concentrations of competing polypeptides in
solution. The antisera were admixed and maintained.
(incubated) with polypeptide P62, D1, D2 or D3 in
solution for a predetermined time sufficient for
immunoreaction (binding) to occur before being added
25   to a microtiter plate coated with P62. The results
of this study with sera from five patients are
summarized in Table 3 below.

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TABLE 3

Concentration of Competing Polypeptides Providing
50% Inhibition of Antibody
Binding to Peptide P62[*]

Sera	Competing Peptide (micrograms per milliliter)			
	P62	D1	D2	D3
TJ	0.05	0.05	0.1	200
VM	0.3	0.3	0.4	3
CV	0.1	0.1	0.1	10
N6	0.6	0.6	0.6	3
JC	1	1	1	500

[*]The ELISA procedures used for these determinations
are described hereinafter in sections IE(2) and IID.

The inhibitory action of polypeptide D1 on
antibody binding to P62 is considered
indistinguishable from the inhibitory effect of P62
itself. This is true for all sera tested.

More interestingly, polypeptide D3 innibited
some of the sera, VM and N6, about as well as tne
larger polypeptides did. This strong inhibitory
effect occurred despite the fact that neither of
those sera showed any binding to D3 bound to the
solid support in the ELISA. These data are believed
to indicate that the polypeptide must be of a certain
minimum length, at least about 15 amino acid
residues, to maintain required secondary structure
when bound to the plastic surface of a microtiter
plate.

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The minimum antigen size necessary for recognition was further studied using polypeptides A5, A6, A7, A8 and A9. As shown in Table 2, polypeptide A5 has five amino acid residues, and each member of the series A6-A9 was increased in length by one amino acid residue over the preceding polypeptide up to the nine residues present in A9. No antiserum tested immunoreacted with these polypeptides when they were bound to microtiter plates in the ELISA described hereinafter.

The data for ability of the A series polypeptides to inhibit binding of human anti-EBNA antibodies to solid phase P62 is shown in Table 4 below.

TABLE 4

Inhibition of Antibody Binding to Peptide
P62 By 100 micrograms per milliliter
Competing Peptide*

| Sera | Percent of Uninhibited Activity | | | | |
	A5	A6	A7	A8	A9
TJ	96	80	91	74	31
VM	93	81	51	17	9
CV	92	93	93	72	20
JC	94	92	60	88	74
S62	86	96	89	95	78
S60	106	109	93	84	53

*These studies were carried out as described for Table 3.

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    Almost all sera tested were inhibited by A9
although very high concentrations were required (more
than 100-fold higher than the concentrations of P62
or D1 needed for equivalent inhibition). In
5   addition, three sera immunoreacted with and were
inhibited by A8 and one by A7. None were inhibited
by the shorter polypeptides A6 and A5.

    Thus, the decrease in immunoreactivity that
parallels a decrease in polypeptide size appears to
10 be due to two effects: (1) the effect of deletion of
the site on the antigen to which the antibody binds
as shown by the A series polypeptides and, (2) the
change in the conformation of the polypeptide as its
size decreases as shown by the D series polypeptides.

15 3. Conformation

    The conformational properties of the
synthetic polypeptides of this invention were studied
using circular dichroism (CD) spectroscopy. The CD
spectra of polypeptides P27, P60, P62, F13, F15 and
20 F16 were determined. The data, partially shown in
Figure 1, indicate that the polypeptides of this
invention which include both of the preferred amino
acid residue sequences; i.e.,

$$-Gly-R^1-Gly-R^2-Gly-$$
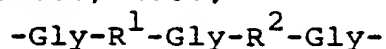
25   wherein R^1 and R^2 are as described
before, and

$$-Gly-Ala-Gly-Gly-Ala-Gly-$$
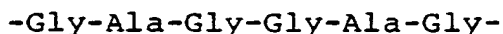
adopt a relatively stable secondary structure or
conformation in a physiological solution at 20°C.
30 Since the predominant conformation of those
polypeptides is relatively stable, it is believed
that human anti-EBNA antibody activity occurs in
response to this particular conformation.

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B. Multimers

The present invention also contemplates a
synthetic multimer containing a plurality of joined
synthetic, random copolymer polypeptide repeating
5   units wherein at least one of the repeating units is
a polypeptide as described herein.

The multimers of this invention, alone or
linked to a carrier, when introduced in an effective
amount into a mammalian host, are capable of inducing
10   the production of antibodies that bind to EBNA.
Those multimers that contain the particularly
preferred synthetic, random copolymer polypeptides of
this invention whose amino acid residue sequences
include both the five residue -Gly-R^1-Gly-R^2-Gly-
15   sequence, wherein R^1 and R^2 are before-defined,
the six residue -Gly-Ala-Gly-Gly-Ala-Gly- sequence,
and also contain at least 50 mole percent glycine
residues are also capable of binding human antibodies
induced by EBNA.

20   Thus, the multimers of this invention, like
their constituent polypeptides, are immunogenic, and
are antigenic to human anti-EBNA antibodies. Those
multimers may therefore be used to induce the
production of anti-EBNA antibodies that are useful in
25   the diagnostic methods and systems discussed
hereinafter, and may also be used as an antigen in
appropriate diagnostic methods and systems.

Multimers that contain fewer than about 35
amino acid residues in the total multimer are
30   typically linked to a carrier for use as an
immunogen. Those multimers that contain more than a
total of about 35 amino acid residues are typically
sufficiently immunogenic to be used without a carrier.

Polypeptide multimers may be prepared by
35   bonding together the synthesized polypeptide monomers

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in a head-to-tail manner using the aforementioned
solid phase method; i.e., one complete polypeptide
sequence can be synthesized on the resin, followed by
one or more of the same or different polypeptide
5   sequences, with the entire multimeric unit thereafter
being cleaved from the resin and used as described
herein. Such head-to-tail polypeptide multimers
preferably contain about 2 to about 4 polypeptide
repeating units.

10   Alternatively, multimers can be prepared as
a polymer of synthetic, random copolymer polypeptides
used as monomers. As used herein, the term "polymer"
in its various grammatical forms is defined as a type
of multimer that contains a plurality of synthetic,
15   random copolymer polypeptide repeating units that are
joined together by other than peptide bonds.

An exemplary polymer of this invention can
be synthesized using the polypeptide monomers of this
invention that contain added cysteine residues at
20   both the amino- and carboxy-termini (diCys
polypeptide). The diCys polypeptide monomers may be
bonded together by intramolecular, interpolypeptide
cysteine disulfide bonds utilizing an oxidation
procedure to form an immunogenic, antigenic polymer.
25   The polymer so prepared contains a plurality of the
synthetic, random copolymer polypeptides of this
invention as repeating units. Those repeating units
are bonded together by the above-discussed oxidized
cysteine (cystine) residues.

30   The presence of one or two terminal Cys
residues in a polypeptide of this invention for the
purposes of binding the polypeptide to a carrier or
for preparing a polymer is not to be construed as
altering the amino acid sequence of polypeptide
35   repeating units of this invention.

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C.   Inocula

In another embodiment, the polypeptides of this invention are used in a pharmaceutically acceptable diluent to form an inoculum or a vaccine that, when administered in an effective amount, is capable of inducing antibodies that immunoreact with EBNA.

The word "inoculum" in its various grammatical forms is used herein to describe a composition containing a polypeptide of this invention as an active ingredient used for the preparation of antibodies against EBNA. When a polypeptide is used to induce antibodies it is to be understood that the polypeptide may be used alone, linked to a carrier or as a multimer, but for ease of expression, these alternatives will not always be expressed hereinafter.

For polypeptides that contain fewer than about 35 amino acid residues, it is preferable to use a carrier for the purpose of inducing the production of antibodies. A polypeptide bound or linked to a carrier will be used illustratively herein where antibodies are being prepared.

The inoculum can be used to produce antibodies for use in a diagnostic assays that detect cells expressing EBNA. The antibodies produced by the inoculum may also be used in a preparation for inducing passive immunity against B lymphocytes expressing EBNA on their cell surfaces.

The word "vaccine" in its various grammatical forms is used herein to describe a type of inoculum containing a polypeptide of this invention as an active ingredient that is used to induce active immunity in a host mammal. Since active immunity involves the production of

antibodies, a vaccine or inoculum may thus contain
identical ingredients, but their uses are different.
In most cases, the ingredients of a vaccine and of an
inoculum are different because many adjuvants useful
5   in animals may not be used in humans.

The present inoculum or vaccine contains an
effective amount of a polypeptide of this invention,
as a multimer such as a polymer of individual
polypeptides linked together through oxidized,
10 polypeptide terminal cysteine residues or as a
conjugate linked to a carrier. However, for ease of
expression, the various embodiments of the
polypeptides of this invention are collectively
referred to herein by the term "polypeptide," and its
15 various grammatical forms.

The effective amount of polypeptide per unit
dose depends, among other things, on the species of
animal inoculated, the body weight of the animal and
the chosen inoculation regimen as is well known in
20 the art. Inocula and vaccines typically contain
polypeptide concentrations of about 10 micrograms to
about 500 milligrams per inoculation (dose). The
stated amounts of polypeptide refer to the weight of
polypeptide without the weight of a carrier, when a
25 carrier is used. Specific, exemplary inocula are
described hereinafter with weight of carrier plus
polypeptide (conjugate) being given.

The term "unit dose" refers to physically
discrete units suitable as unitary dosages for
30 animals, each unit containing a predetermined
quantity of active material calculated to produce the
desired therapeutic effect in association with the
required diluent; i.e., carrier, or vehicle. The
specifications for the novel unit dose of this
35 invention are dictated by and are directly dependent

on (a) the unique characteristics of the active
material and the particular therapeutic effect to be
achieved, and (b) the limitations inherent in the art
of compounding such active material for therapeutic
5   use in animals, as disclosed in detail in the
specification, these being features of the present
invention.

Inocula are typically prepared from the
dried solid polypeptide-conjugate or polypeptide
10 polymer by suspending the polypeptide-conjugate or
polypeptide polymer in a physiologically tolerable
(acceptable) diluent such as water, saline or
phosphate-buffered saline.

Inocula may also include an adjuvant.
15 Adjuvants such as complete Freund's adjuvant (CFA),
incomplete Freund'S adjuvant (IFA) and alum are
materials well known in the art, and are available
commercially from several sources.

D.   Receptors

20 Antibodies and substantially whole
antibodies raised to (induced by) the polypeptides of
this invention as well as antibody combining sites
prepared from such antibodies constitute still
another embodiment of this invention. These
25 molecules are collectively referred to herein as
receptors. Receptors are raised in mammalian hosts
such as mice, rabbits, horses and the like by
immunization using the inocula described hereinabove.

Suitable monoclonal receptors, typically
30 whole antibodies, may also be prepared using
hybridoma technology described by Niman et. al.,
Proc. Natl. Sci., U.S.A., 80:4949-4953 (1983), which
description is incorporated herein by reference.
Briefly, to form the hybridoma from which the
35 monoclonal receptor is produced, a myeloma or other

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self-perpetuating cell line is fused with lymphocytes
obtained from the spleen of a mammal hyperimmunized
with a polypeptide of this invention.

It is preferred that the myeloma cell line
5   be from the same species as the lymphocytes.
Typically, a mouse of the strain 129 G1x$^+$ is the
preferred mammal. Suitable mouse myelomas for use in
the present invention include the
hypoxanthine-aminopterin-thymidine-sensitive (HAT)
10   cell lines P3X63-Ag8.653 (ATCC CRL 1580), and
Sp2/0-Ag14 (ATCC CRL 1581).

Spleenocytes are typically fused with
myeloma cells using polyethylene glycol (PEG) 1500.
Fused hybrids are selected by their sensitivity to
15   HAT. Hybridomas producing the receptor molecules of
this invention are identified using the enzyme linked
immunosorbent assay (ELISA) described in the
Materials and Methods section II D hereinafter.

Monocloncal receptors need not only be
20   obtained from hybridoma supernatants, but may also be
obtained in generally more concentrated form from
ascites fluid of mammals into which the desired
hybridoma has been introduced. Production of
monoclonal antibodies using ascites fluid is well
25   known and will not be dealt with further herein.

A receptor of this invention binds both to
the polypeptide to which it was raised and also binds
to the corresponding EBNA antigenic determinant site
the polypeptide of this invention immunologically
30   mimics. Thus, a polypeptide of this invention may be
both an immunogen and an antigen.

The receptors of this invention may be
described as being oligoclonal as compared to
naturally occurring polyclonal antibodies since they
35   are raised to an immnogen having relatively few

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epitopes as compared to the epitopes of an intact
EBNA molecule. Consequently, receptors of this
invention bind to epitopes of the polypeptide while
naturally occurring antibodies raised to EBNA bind to
5   epitopes throughout the EBNA molecule.

Exemplary receptor molecules containing
antibody combining sites of this invention raised in
rabbits to the polypeptides shown in Table 1 were
studied using the immunoblotting procedures of
10   Towbin, et. al., Proc. Natl. Acad. Sci., U.S.A.,
76:4350-4354 (1979) and Billings, et. al., Proc.
Natl. Acad. Sci., U.S.A., 80:7104-7108 (1983).
Further details are provided in the Materials and
Methods section (II).
15   It was found that all the polypeptides of
this study elicited rabbit anti-polypeptide
antibodies when linked to a protein carrier as a.'
conjugate and introduced in an effective amounts into
rabbit hosts in an inoculum as described
20   hereinafter. These receptor molecules recognized
intact EBNA protein isolated from the EBV-transformed
human B lymphoblast cell lines WI-L2, Raji and
Daudi. Data from these studies are partially shown
in Figure 2. In control studies, protein extracts of
25   B lymphocytes negative for EBV infection (BJAB cells;
available at the Scripps Clinic and Research
Foundation, La Jolla, CA) failed to yield reactive
bands with the anti-polypeptide antisera. These data
indicate that exemplary receptor molecules of this
30   invention immunoreact with an EBV infection-specific
protein.

In addition, it was found that the
immunoreactivity of the rabbit anti-polypeptide
antibodies for intact EBNA protein could be blocked
35   by the inducing, immunogenic polypeptide used as an

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antigen, as is also shown in Figure 2. These results
demonstrate that the idiotypes (antibody-combining
sites) of the anti-polypeptide antisera were specific
for EBNA antigenic determinants.

5   The rabbit anti-polypeptide antibody to
polypeptide P62 was used in a competition study to
examine the antigenic relatedness of polypeptides
P27, P62, P60 and P89. This antibody cross-reacted
extensively with the conjugated and non-conjugated
10   polypeptides in the ELISA described hereinafter. The
binding of anti-polypeptide P62 to polypeptide P62 in
the solid phase was inhibited 98 percent by first
incubating the antibody with polypeptide P62. In the
same manner, the binding of anti-polypeptide P62 to
15   polypeptide P62 was inhibited 81% by polypeptide P60,
and 36% by polypeptide P27. Polypeptide P89 did not
inhibit anti-polypeptide P62 reactivity at all.

               To determine whether or not the antibodies
in human EBV-immune serum also recognized this
20   antigenic determinant, a competition study was
performed using the serum of an EBV-immune,
rheumatoid arthritis patient (serum 1011). The
results, shown in Figure 3, were similar to those
obtained when anti-polypeptide-P62 was used. This
25   indicates that tne antigenic determinant shared by
polypeptides P27, P62 and P60 mimics a naturally
occuring immunogenic determinant of EBNA.

E.   Diagnostic Assays Systems and Methods
               The polypeptides, antibodies and antibody
30   combining sites (receptors) raised to the before
described polypeptides, and methods of the present
invention may also be used for diagnostic tests, such
as immunoassays. Such diagnostic techniques include,
for example, enzyme immune assay, enzyme multipled
35   immunoassay technique (EMIT), enzyme-linked

immunosorbent (ELISA), radio-immune assay (RIA),
flourescence immune assay, either single or double
antibody techniques, and other techniques in which
either the receptor or the antigen is labeled with
5   some detectable tag or indicating means. See
generally Maggio, Enzyme Immunoassay, CRC Press,
Cleveland, Ohio (1981); and Goldman, M., Flourescent
Antibody Methods, Academic Press, New York, N.Y.
(1980). Specific examples of such assay methods and
10   systems useful in carrying out those methods are
discussed hereinbelow.

        1.   Assays For EBNA
        A method for assaying for the presence of
EBNA in a body sample is also contemplated herein.
15   In a general method, a body sample to be assayed is
provided, and is admixed with receptor molecules that
contain an antibody combining site raised to a
synthetic, random copolymer polypeptide of this
invention. The admixture is maintained for a
20   predetermined period of time sufficient for the   ·
receptor molecules to immunoreact with EBNA present
in the body sample. The amount of that
immunoreaction is then measured to determine whether
EBNA molecules were present or absent in the assayed
25   body sample.

        An illustrative diagnostic system in kit
form embodying one aspect the present invention that
is useful for detecting EBNA present in an aliquot of
a body sample contains receptor molecules of this
30   invention such as antibodies, substantially whole
antibodies, or antibody combining sites like Fab and
$F(ab')_2$ antibody portions raised to a polypeptide
of this invention in one package. This system also
includes an indicating means for signaling the

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presence of an immunoreaction between the receptor and the antigen.

Typical indicating means include radioisotopes such as ^{125}I and ^{131}I, enzymes such
5   as alkaline phosphatase, horseradish peroxidase, beta-D-galactosidase and glucose oxidase, and fluorochrome dyes such as fluorescein and rhodamine. The indicating means may be linked directly to receptor of this invention. The indicating means may
10 also be linked to a separate molecule such as to a second antibody, to an antibody combining site or to Staphylococcus aureus (S. aureus) protein A that reacts with (binds to) the receptor of this invention. A specific example of such a separate
15 molecule indicating means is ^{125}I-labeled S. aureus protein A.

The indicating means permits the immunoreaction product to be detected, and is packaged separately from the receptor when not linked
20 directly to a receptor of this invention. When admixed with a body sample such as an acetone-fixed peripheral blood lymphocyte (PBL) smear, the receptor molecule immunoreacts with the EBNA to form an immunoreactant, and the indicating means present then
25 signals the formation of immunoreaction product.

One embodiment of an EBNA diagnostic method is an immunoflourescent assay that includes an amplifying reagent. In such an assay a PBL smear is acetone-fixed to a plain microscope slide. An
30 aliquot of antibodies raised in accordance with this invention, e.g., raised in rabbits, generally about 10 micrograms to about 500 micrograms, is contacted with the slide using well-known techniques.

After rinsing away any un-immunoreacted
35 antibodies of this invention, any non-specific

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binding sites on the slide are typically blocked with
a protein such as bovine serum albumin (BSA), if
desired. A second reagent (amplifying reagent) such
as complement, or anti-immunoglobulin antibodies,
5   e.g., guinea pig complement, can then be incubated on
the test slide.

        After this second incubation, any unreacted
of the amplifying reagent is removed as by rinsing
leaving only that which is bound to the first-named
10   antibodies on the assay slide. A third reagent
(indicating means), e.g., antibody, like goat
anti-guinea pig complement, is then incubated on the
test slide. The third reagent is labeled by being
linked to a flourochrome dye such as fluorescein
15   isothiocyanate (FITC), rhodamine B isothiocyanate
(RITC), tetramethylrhodamine isothiocyanate (TRITC),
4, 4'-diisothiocyanostilbene-2,2'-disulfonic acid
(DIDS), and the like as are well known in the art.

        Any unreacted third reagent is rinsed off
20   after this third incubation, leaving any FITC labeled
goat-antiguinea pig complement antibodies that bind
to the complement on the test slide. The presence of
the FITC labeled third reagent may be detected using
flourescence microscopy and thereby signal the
25   presence of EBV infection.

        B lymphocytes known to be infected with EBV
were tested for the presence of EBNA using the
immunoflourescence assay method described above and
in more detail in the Materials and Methods section
30   II. Rabbit antibodies raised to each of the
polypeptides shown in Table 1 were able to detect
EBNA in the EBV infected cell line WI-L2.

        A preferred diagnostic system, preferably in
kit form, useful for carrying out the above assay
35   method includes, in separate packages, (a) receptors

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(antibodies) of this invention that immunoreact with
EBNA, (b) a second, amplifying reagent such as
complement, like guinea pig complement,
anti-immunogloulin antibodies or $\underline{S}$. $\underline{aureus}$ protein A
5   that reacts with the receptor, and (c) an indicating
means that may be linked directly to the amplifying
means or may be a portion of a separate molecule such
as an antibody or antibody-portion that reacts with
the amplifying reagent. The indicating means
10   indirectly signals the immunoreaction of the receptor
molecule and EBNA through the mediation of the
amplifying reagent.

        Receptor molecules and separate indicating
means of any diagnostic system described herein, as
15   well as the above-described amplifying reagant, may
be provided in solution, as a liquid dispersion or as
a substantially dry powder, e.g., in lyophilized form.
Where the indicating means is a separate molecule
from the amplifying reagent, it is preferred that the
20   indicating means be packaged separately. Where the
indicating means is an enzyme, the enzyme's substrate
may also be provided in a separate package of the
system. A solid support such as the before-described
microscope slide, one or more buffers and acetone may
25   also be included as separately packaged elements in
this diagnostic assay system.

        The packages discussed herein in relation to
diagnostic systems are those customarily utilized in
diagnostic systems. Such packages include glass and
30   plastic (e.g., polyethylene, polypropylene and
polycarbonate) bottles, vials, plastic and
plastic-foil laminated envelopes and the like.

        The use of whole, intact, biologically
active antibodies is not necessary in many diagnostic
35   systems such as the immunoflourescent assay described

above. Rather, only the immunologically active,
idiotype-containing, antigen binding and recognition
receptor site; i.e., the antibody combining site, of
the antibody molecule may be used. Examples of such
5   antibody combining sites are those known in the art
as Fab and F(ab')$_2$ antibody portions that are
prepared by proteolysis using papain and pepsin,
respectively, as is well known in the art.

2.   Assays For Anti-EBNA Antibodies

10   Another diagnostic method of this invention
is an ELISA that detects anti-EBNA antibodies in a
body sample. Here, a particularly preferred
polypeptide of this invention such as polypeptide P62
is used as an antigen, and is preferably bound on
15   (adsorbed to) or otherwise linked to a solid matrix
such as the cross-linked dextran available under the
trademark SEPHADEX from Pharmacia Fine Chemicals
(Piscataway, New Jersey), agarose, beads of glass,
polyvinyl chloride, polystyrene, cross-linked
20   acrylamide, nitrocellulose or the wells of a
microtiter plate to form a solid support.

The particularly preferred polypeptide is
admixed with a provided body sample to be assayed.
The admixture is maintained for a predetermined time
25   sufficient for anti-EBNA antibodies present in the
body sample to immunoreact with the polypeptide. The
presence of that immunoreaction is then determined as
with an indicting means to signal the presence of
anti-EBNA antibodies in the assayed body sample.

30   An exemplary ELISA utilizing the above
method uses a solid support comprised of a
particularly preferred polypeptide of this invention
adsorbed onto a solid matrix comprised of the wells
of a twelve or ninety-six well microtiter plate made
35   of polystyrene or polyvinyl chloride. Non-specific

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binding sites on the microtiter well walls are
thereafter typically blocked with a protein such as
bovine serum albumin (BSA). Unbound polypeptide and
BSA are removed from the microtiter well as by
5   rinsing.

A body sample aliquot such as human serum,
blood or plasma is admixed with the above-described
polypeptide-bound solid support to form an admixture
containing solid and liquid phases. The solid-liquid
10   phase admixture is maintained for a time sufficient
for anti-EBNA antibodies in the body sample to
immunoreact with the polypeptide antigen. The solid
and liquid phases are thereafter generally separated.

A solution of a second, labeled, indicating
15   means-containing antibody, antibody combining site or
S. aureus protein A that reacts with the first-named
antibody is then admixed with the solid phase to form
another solid-liquid phase admixture. An exemplary
second antibody is a peroxidase-labeled goat
20   anti-human Ig antibody where the first-named
antibodies are from a human body sample. Additional,
useful enzyme labels include alkaline phosphase,
beta-D-galactosidase and glucose oxidase. The
admixture formed from the solid phase and the second
25   labeled antibody solution is maintained (incubated)
for a predetermined time period (e.g., 30 minutes)
sufficient to form a reactant between the first-named
antibody and the indicating means such as an
immunoreaction between the two antibodies. The solid
30   and liquid phases are thereafter separated.

The second antibody described above may also
be specific for and immunoreact with only one of the
classes of immunoglobulin (e.g., IgG, IgM, IgE, IgA,
or IgD). Such antibodies provide the ability to
35   identify the immunoglobulin class of anti-EBNA

antibody present in the body sample, as is shown in
Table 6, hereinafter.   In addition, the second
antibody or antibody combining site may be specific
for and immunoreact with only one of the two types of
5   immunoglobulin light chains (e.g., kappa or lambda).
These antibodies provide the ability to identify the
isotype of the immunoglobulin molecule present in the
body sample.

         A solution containing a substrate for the
10   enzyme label such as hydrogen peroxide for peroxidase
and a color-forming dye precursor such as
o-phenylenediamine, or p-nitrophenyl phosphate for
alkaline phosphatase is thereafter admixed with the
solid phase. The optical density at a preselected
15   wavelength (e.g., 490 or 405 nanometers,
respectively) may then be determined after a
predetermined time period has elapsed (e.g., 60
minutes), and compared to the optical density of a
control to determine whether anti-EBNA antibodies
20   were present in the body sample.

         Another embodiment of this invention
comprises a diagnostic system in kit form that
includes a solid support comprised of a solid matrix
such as a polystyrene twelve-well microtiter strip,
25   and a polypeptide of this invention, absorbed (bound)
or otherwise affixed to the solid matrix to form a
solid matrix. This system preferably also includes
separately packaged anti-human Ig antibodies having a
linked indicating means such as peroxidase-labeled
30   goat anti-human Ig antibodies, and may also include
substrate for the linked indicating means such as
hydrogen peroxide and a color forming due precursor
such as o-phenylenediamine, in further, separate
packages. Hydrogen peroxide is typically not
35   included in the kit due to its relative instability,

and is typically supplied by the end user. Buffer
salts useful in an assay utilizing this system may
also be included in one or more separate packages in
dry or liquid form. Separate packages containing
5   human anti-EBNA antibodies and human antibodies free
from anti-EBNA antibodies (normal human antibodies)
may also be included as positive and negative
controls, respectively. An assay for the presence of
anti-EBNA antibodies in a body sample such as serum
10   may be carried out with this diagnostic system using
the above-described method.

     An exemplary ELISA, similar to that
described before and described in detail in the
Materials and Methods section (II) hereinafter, was
15   used to screen for the presence of anti-polypeptide
immunoglobulin in the sera of 91 people with
anti-EBNA positive serotypes established using the
anti-complement immunofluorescence (ACIF) described
hereinbefore. When the sera were assayed at a 1:20
20   dilution, all 91 were positive against (bound to)
polypeptides P27, P62, P60 and F16., F14 and F15.
Even at the higher dilution of 1:320, 83 of the 91
EBNA positive sera immunoreacted with polypeptide P62
in the ELISA. Thus, there appears to be an excellent
25   correlation between the anti-EBNA antibody titer
established by ACIF and the antipeptide activity of
each serum.

     In addition, the results illustrate an
excellent correlation between the ACIF method and the
30   present ELISA technique, which is simpler and easier
to use. Still further, the results illustrate the
usefulness of polypeptides of this invention for a
diagnostic assay for anti-EBNA antibodies.

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Table 5, below, shows the reactivities of sera obtained from two subjects before and after contracting EBV-induced infectious mononucleosis (IM). In both cases antibodies that bind to
5   polypeptides P27, P62 and P60 were absent before infection, but appeared afterward. In contrast, no antibodies were produced by either of these individuals that bound to polypeptide P89.

In further study, stored sera from a
10   previously reported panel of 27 EBV non-immune donors [Catalano et. al., J. Clin. Invest., 65:1238-1245 (1980)] were screened for binding to polypeptides P62 and P60 in the before-described ELISA. The EBV-immune status of the sera used was defined by the
15   presence or absence of Epstein-Barr viral capsid antigen (VCA). Individuals who have no serum antibodies to VCA (VCA-) have never been infected with EBV. VCA positive (VCA+) individuals have had EBV infections and typically carry a low level of
20   anti-EBNA antibodies. None of the sera displayed significant reactivity to either polypeptide as is seen from Table 5, below.

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Table 5

ANTIBODIES TO EBNA PEPTIDES IN HUMAN SERA[1]

PATIENT	$OD_{405} \times 10^3$ obtained[2] with Patient Antibodies to:			
	P27	P62	P60	P89
VCA+ Normal 1[3]	155	958	154	23
VCA+ Normal 2[3]	516	819	145	16
SB pre-mononucleosis	11	13	51	9
SB post-mononucleosis	33	514	115	23
MV pre-mononucleosis	15	77	72	29
MV post-mononucleosis	107	631	162	25
VCA-Normals (n=27)[4]	67±23[5]	ND[6]	45±18	ND

1   All sera were tested at a dilution of 1:20.
2   Optical density measured at 405 nanometer light
    wavelength after 30 minute substrate incubations.
3   VCA+ sera from individuals showing no clinical signs of
    present EBV related disease.
4   Sera from 27 individuals showing no clinical signs of
    EBV related disease, past or present, as indicated by
    the absence of antibodies to VCA.
5   Average optical density ± one standard deviation.
6   Not done.

        To examine the correlation between the ELISA
and the ACIF diagnostic technique through the course
of infection, stored sera from 8 college-age
students, collected sequentially after onset of
infectious mononucleosis (IM), were examined in the
before-described ELISA using polypeptide P62.   All
students developed anti-EBNA titers from 1 month to 1
year following infection as measured by the classical

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method; i.e., ACIF. Henle, et. al., J. Infect.
Dis.,130:231- 239, (1974).

          In one-half of the subjects, anti-P62
antibodies rose in parallel with the corresponding
5   anti-EBNA titer as is shown for patient 15 in Figure
4. In the other half, antibodies against polypeptide
P62 were detected in the first month after the onset
of symptoms while those antibodies were detected at
later times using the ACIF technique. These results
10   are shown for patients 14 and 2 in Figure 5.
Anti-P62 antibodies were therefore detectable using
the ELISA of the present invention before anti-EBNA
antibodies were detectable by the standard
anti-complement immunoflourescence assay.
15   To differentiate the class of immunoglobulin
predominantly responsible for an individual's immune
response at a given time during the course of
infectious mononucleosis (IM), secondary (indicating)
antibodies specific for human IgG or IgM were used in
20   the ELISA as described in the Methods and Materials
section (II). Table 6, below, shows the results of
this study with the sera of two individuals from
different points in time during EBV infection
measured against polypeptide P62 in the ELISA.

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Table 6

POLYPEPTIDE P62-BOUND ELISA-DETECTED
APPEARANCE OF ANTIPEPTIDE ACTIVITY
AFTER MONONUCLEOSIS INFECTION

Time After Infection	Patient MV		Patient 15	
	IgM[1]	IgG[2]	IgM[1]	IgG[2]
Pre Infection	103[3]	74	ND[4]	ND
1 Week	245	80	152	113
1 Month	87	37	225	118
3 Months	136	60	208	118
12 Months	ND	ND	249	375
21 Months	145	600	185	994

1 Patient IgM level detected using a second antibody
   specific for human IgM.
2 Patient IgG level detected using a second antibody
   specific for human IgG.
3 Optical density measured at 405 nanometer light
   wavelength.
4 Not done.

Using this ELISA method, the rise in IgM
antibody values, although small, are repeatable and
found in all sequential sera tested. The immune
response measured by the ELISA assay is normal in
that IgM classically appears before IgG during EBV
infection. The appearance of IgM antibodies prior to
IgG antibodies is particularly well shown for patient
15 in Table 6. The above results again show that
infection with EBV causes the production of

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antibodies that react with at least one polypeptide of this invention.

In a larger anti-polypeptide ELISA study, the panel of 19 VCA- sera described hereinbefore were
5   screened against polypeptides P27, P62, P60, F12, F13, F14, F15 and F16. None of the VCA- sera were found to react positively. Typical data are shown in Table 7, below. Sera from clinically normal individuals positive for VCA antibodies were tested
10   at two dilutions. Typical results, shown in Table 7 below, indicate that all of the VCA+ individuals were positive for; i.e., had antibodies that bound to, each of the polypeptides.

The sera of a number of rheumatoid arthritis
15   (RA) patients were also screened in the ELISA. These results are also summarized in Table 7.

Table 7

ELISA-DETERMINED

20   AVERAGE ANTIPEPTIDE LEVELS IN HUMAN SERA

Patient Group	Number of Sera		Average Antibody Activity to Polypeptides[1]					
			P27	P62	P60	F12	F13	F16
Nor-[2]	1/20[3]	19	22	17	77	34	41	47
Nor+[4]	1/20	26	521	854	394	128	70	733
Nor+	1/320	48	90	223	70	37	37	166
RA+[5]	1/20	28	597	999	564	118	113	843
RA+	1/320	48	126	348	122	50	50	231

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1   Activity measured as optical density at 405
    nanometers light wavelength after 30 minutes of
    serum incubation.

2   VCA negative individuals.

5   3   Dilution at which sera were tested.

4   VCA positive individuals.

5   Individuals diagnosed as having rheumatoid
    arthritis.

10   The difference between the normal, VCA
    positive (control) and RA patients can best be seen
    at the serum dilution 1:320. The antibody levels in
    RA patients were significantly higher for every
    polypeptide tested at this dilution when analyzed
15 using the Wilcox Rank Sum method (significance level
    greater than 99%).
            Patients with Sjogren's Syndrome, Systemic
    Lupus Erythematosus (SLE) and Progressive Systemic
    Schlerosis (PSS) were also screened at both high and
20 low serum dilutions. The only differences found
    between these patient groups and normals was a
    relatively higher average titer of the PSS patients
    to polypeptides P27, P62, P60, F16, F14 and F15.
    These results are believed to possibly be due to a
25 previous EBV related infection or the involvement of
    EBV in those autommune diseases. These data do not
    detract from the usefulness of the ELISA as a
    diagnostic method.
            3.   Preparation For Passive Immunization
30   A patient with latently infected B
    lymphocytes that express EBNA on their cell surfaces
    can be treated with receptors of this invention,
    preferably as whole antibodies, raised to the
    synthetic polypeptides of the present invention that
35 immunoreact with EBNA. The receptors are

administered in a unit dose having an effective
amount of receptors dispersed in a pharmaceutically
acceptable diluent.

5   An effective amount of such antibodies
varies depending on the reactivity and type of the
antibodies. Generally, about 0.5 milligrams to about
25.0 milligrams of antibody per kilogram patient body
weight is considered effective. The antibodies can
be administered intravenously, intramuscularly, or
10   intraperitoneally, with several administrations given
at 3 to 20 day intervals. The antibodies can also be
given in conjunction with surgical or chemical
treatment.

The antibodies can be obtained from the sera
15   or plasma of an animal species different from tne
patient to be treated by raising antibodies to the
polypeptide of this invention using the
before-described inocula. The antibodies can also be
obtained from monoclonal sources such as ascites
20   fluid by preparing a hybridoma cell line using
techniques known in the art. Whole antibodies are
preferred as the combining site since they are
capable of activating the complement system when an
immune complex is formed.

25   II. METHODS AND MATERIALS

A. Synthesis of Polypeptides

The polypeptides of this invention were
chemically synthesized by solid-phase methods as
described in Merrifield et. al., J. Am. Chem. Soc.,
30   85:2149-2154 (1963) and Houghten et. al., Int. J.
Pept. Prot. Res. 16:311-320 (1980). The solid phase
method of polypeptide synthesis was practiced
utilizing a Beckman Model 990B Polypeptide
Synthesizer, available commercially from Beckman
35   Instrument Co., Berkeley, CA, U.S.A.

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For polypeptides having fewer than 35
residues that were used in inocula, a cysteine
residue was added to the amino-terminus or to the
carboxyl-terminus to assist in coupling to a protein
5   carrier as described below. The compositions of all
polypeptides were confirmed by amino acid analysis.

In preparing a synthetic polypeptide of this
invention by the above solid phase method, the amino
acid residues are linked to a resin (solid phase)
10   through an ester linkage from the carboxy-terminal
residue. When the polypeptide is to be linked to a
carrier via a Cys residue or polymerized via terminal
Cys residues, it is convenient to utilize that Cys
residue as the carboxy-terminal residue that is
15   ester-bonded to the resin.

The alpha-amino group of each added amino
acid is typically protected by a
tertiary-butoxycarbonyl (t-BOC) group prior to the
amino acid being added into the growing polypeptide
20   chain. The t-BOC group is then removed prior to
addition of the next amino acid to the growing
polypeptide chain.

Reactive amino acid side chains were also
protected during synthesis of the polypeptides.
25   Usual side-chain protecting groups were used for the
remaining amino acid residues as follows:
O-(p-bromobenzyloxycarbonyl) for tyrosine; O-benzyl
for threonine, serine, aspartic acid and glutamic
acid; S-methoxybenzyl for cysteine, dinitrophenyl for
30   histidine; 2-chlorobenzoxycarbonyl for lysine and
tosyl for arginine.

Protected amino acids were recrystallized
from appropriate solvents to give single spots by
thin layer chromatography. Couplings were typically
35   carried out using a ten-fold molar excess of both

protected amino acid and dicyclohexyl carbodiimide
over the number of milliequivalents of initial
N-terminal amino acid. A two molar excess of both
reagents may also be used. For asparagine, an equal

5   molar amount of N-hydroxy-benzotriazole was added to
the protected amino acid and dimethyl formamide was
used as the solvent. All coupling reactions were
more than 99% complete by the picric acid test of
Gisin, Anal. Chem. Acta. 58:248-249 (1972).

10   After preparation of a desired polypeptide,
a portion of the resulting, protected polypeptide
(about 1 gram) was treated with two milliliters of
anisole, and anhydrous hydrogen flouride, about 20
milliliters, was condensed into the reaction vessel

15   at dry ice temperature. The resulting mixture was
stirred at about 4 degrees C. for about one hour to
cleave the protecting groups and to remove the
polypeptide from the resin. After evaporating the
hydrogen flouride at a temperature of 4 degrees C.

20   with a stream of N_2, the residue was extracted with
anhydrous diethyl ether three times to remove the
anisole, and the residue was dried in vacuo.

             The vacuum dried material was extracted with
5% aqueous acetic acid (3 times 50 milliliters) to

25   separate the free polypeptide from the resin. The
extract-containing solution was lyophilized to
provide a monomeric unoxidized polypeptide.

             The produced synthetic polypeptide may be
used as a reagent in an exzyme-linked immunosorbent

30   assay (ELISA) to detect anti-EBNA antibodies. The
synthetic polypeptide may also be used to produce an
inoculum, usually by linking it to a carrier to form
conjugate and then dispersing an effective amount of
the conjugate in a physiologically tolerable diluent,

35   as is discussed hereinafter.

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It is also to be noted that a synthetic multimer of this invention can be prepared by the solid phase synthesis of a plurality of the polypeptides of this invention linked together

5   end-to-end (head-to-tail) by an amide bond between the carboxyl-terminal residue of one polypeptide and the amino-terminal residue of a second polypeptide. Such synthetic multimers are preferably synthesized as a single long polypeptide multimer, but can also

10 be prepared as individual polypeptides that are linked together subsequent to their individual syntheses, using a carbodiimide reagent such as 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride in water. The total

15 number of amino acid residues contained in a multimer prepared as a single polypeptide chain is preferably less than about 50, so that up to about eight polypeptides of this invention can be incorporated into a single head-to-tail multimer chain that is

20 synthesized as a single polypeptide. A synthetic head-to-tail multimer more preferably contains two to about four blocks of linked, synthetic, random copolymer polypeptides of this invention, and a total of less than about 40 amino acid residues.

25 B. Preparation of Polymers

The polypeptides of the present invention may be connected together to form an antigenic and/or immunogenic polymer (synthetic multimer) comprising a plurality of the polypeptide repeating units. Such a

30 polymer typically has the advantage of increased immunogenicity and antigenicity. In addition, a carrier is typically not needed when a polymeric immunogen is utilized. Where different polypeptide monomers are used to make up the polymer, the ability

35 to immunoreact with antibodies to several EBNA

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antigenic determinants is obtained. A still further
advantage is the ability of such a polymer when used
in an inoculum to induce antibodies that immunoreact
with several antigenic determinants of EBNA.

5   A polymer of this invention may be prepared
by synthesizing the polypeptides as discussed above
and including cysteine residues at both the amino-
and carboxy-termini to form a "diCys-terminated"
polypeptide. For example, each of the polypeptides
10   of Table 1 and polypeptides D1 and D2 of Table 2 may
be synthesized to contain an additional Cys residue
at each of the amino- and carboxy-termini to provide
diCys-terminated polypeptides in their reduced
forms. After synthesis, in a typical laboratory
15   preparation, 10 milligrams of the diCys polypeptide
(containing cysteine residues in un-oxidized form)
are dissolved in 250 milliliters (ml) of 0.1 molar
(M) ammonium bicarbonate buffer. The dissolved
diCys-terminated polypeptide is then air oxidized by
20   stirring the resulting solution gently for a period
of about 18 hours in the air, or until there is no
detectable free mercaptan by the Ellman test. [See
Ellman, Arch. Biochem. Biophys., 82:70-77 (1959).]
        The polymer (synthetic multimer) so prepared
25   contains a plurality of the synthetic, random
copolymer polypeptide repeating units that are bonded
together by oxidizing cysteine (cystine) residues.
Such polymers typically contain their polypeptide
repeating units bonded together in a head-to-tail
30   manner as well as in head-to-head and tail-to-tail
manners; i.e., the amino-termini of two polypeptide
repeating units may be bonded together through a
single cystine residue as may two carboxyl-termini
since the linking groups at both polypeptide termini
35   are identical.

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C. Coupling To Carriers

        The synthetic polypeptides were coupled to
keyhole limpet hemocyanin (KLH) as carrier by the
method described in Liu et al., Biochem., 80:690
5   (1979). Briefly, 4 milligrams (mg) of the carrier
were activated with 0.51 mg of m-maleimidobenzoyl-
N-hydroxysuccinimide ester, and were subsequently
reacted with 5 mg of the polypeptide through an
amino- or carboxy-terminal cysteine to provide a
10   conjugate containing about 10 to about 35 percent by
weight polypeptide.

        One or more additional amino acid residues
may be added to the amino- or carboxy- termini of the
synthetic polypeptide to assist in binding the
15   polypeptide to a carrier. As discussed before,
cysteine residues added at the amino- or
carboxy-termini of the synthetic polypeptide have
been found to be particularly useful for forming
polymers via disulfide bonds. However, other methods
20   well known in the art for preparing conjugates can
also be used. Exemplary additional linking
procedures include the use of Michael addition
reaction products, dialdehydes such as
glutaraldehyde, Klipstein et al., J.Infect. Dis.,
25   147:318-326 (1983) and the like, or the use of
carbodiimide technology as in the use of a
water-soluble carbodiimide to form amide links to the
carrier, as discussed before for linking a plurality
of polypeptides together to form a synthetic multimer.
30   Useful carriers are well known in the art,
and are generally proteins themselves. Exemplary of
such carriers are keyhole hemocyanin (KLH), edestin,
thyroglobulin, albumins such as bovine serum albumin
(BSA) or human serum albumin (HSA), red blood cells
35   such as sheep erthrocytes (SRBC), tetanus toxoid,

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cholera toxoid as well as polyamino acids such as poly (D-lysine:D-glutamic acid), and the like.

As is also well known in the art, it is often benefical to bind a synthetic polypeptide to
5   its carrier by means of an intermediate, linking group. As noted above, glutaraldehyde is one such linking group. However, when cysteine is used, the intermediate linking group is preferably an m-maleimidobenxoyl N-hydroxy succinimide (MBS), as
10   was used herein.

Additionally, MBS may be first added to the carrier by an ester-amide interchange reaction as disclosed by Liu et al., supra. Thereafter, the addition can be followed by addition of a blocked
15   mercapto group such as thiolacetic acid (CH_3COSH) across the maleimido-double bond. After cleavage of the acyl blocking group, a disulfide bond is formed between the deblocked linking group mercaptan and the mercaptan of the added cysteine residue of the
20   synthetic polypeptide.

The choice of carrier is more dependent upon the ultimate use of the immunogen than upon the determinant portion of the immunogen, and is based upon criteria not particularly involved in the
25   present invention. For example, if a inoculum is to be used in animals, a carrier that does not generate an untoward reaction in the particular animal should be selected.

D.   ELISA
30   Anti-peptide antibody binding and inhibition studies were carried out by an enzyme-linked immunosorbent assay (ELISA) as described below.

Briefly, microtiter wells (Costar, #3590, Cambridge, MA) were coated with individual
35   polypeptides as antigens by adding 100 microliters

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(ul) of BBS [10 millimoler (mM) sodium borate (pH
8.3), 150 mM NaCl] containing polypeptide at a
concentration of 10 micrograms per milliliter
(ug/ml). Contact between the wells and

5   antigen-containing solution was maintained for a
predetermined time, typically 15 minutes, and at 20
degrees C., to form an antigen-coated solid phase.
The solid and liquid phases were separated and the
wells were washed three times with BBS.

10   Non-specific binding sites were blocked by
admixing 200 microliters of 1% bovine serum albumin
(BSA) in each well to form another solid-liquid phase
admixture, and maintaining that solid-liquid phase
admixture for 30 minutes, at 20 degrees C. The

15   phases were separated and excess, unbound BSA was
removed by washing three times with BBS.

Rabbit and human sera (body sample aliquots)
were assayed for anti-polypeptide activity by adding
100 microliters of a serum diluted 1:20 in BBS per

20   well to form a solid/liquid phase composition.
Contact between the diluted sera and the
antigen-coated solid phase was maintained for a
predetermined time such as 1 hour, and at 20 degress
C., for an immunoreactant to form. The solid and

25   liquid phases were separated, and the solid phase;
i.e., antigen-coated, immunoreactant-
containing wells, was then washed three times with
BBS.

The antibodies in human sera that
30   immunoreacted with an adsored polypeptide were
detected using an indicating means comprising
alkaline phosphatase-conjugated goat anti-human Ig
antibody (Tago, Burlington, CA). The antibodies in
rabbit sera that immunoreacted with an adsorbed
35   polypeptide were detected using an indicating means

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comprising alkaline phosphatase-conjugated goat
anti-rabbit Ig antibody (Kirkegard & Perry
Laboratories, Inc., Gaithersburg, MD). In either
instance, 100 microliters of the indicating antibody
5   diluted 1:300 in BBS were added per well to form a
further solid-liquid phase composition. This solid-
liquid phase composition was maintained for a
predetermined time, one hour, for the formation of a
reaction product between the human antibodies bound
10   to the solid phase and the indicating means, and at
20 degrees C. The phases were separated, and the
solid phase was washed 3 times with BBS.
        Alkaline phosphatase-conjugated antibody
bound to polypeptide specific antibody was detected
15   by spectrophotometrically measuring the enzymatic
hydrolysis of p-nitrophenyl phosphate to
p-nitrophenol. Briefly, 100 microliters of
p-nitrophenyl phosphate [1 milligram per milliliter
in 2 mM MgCl 2, (pH 9.8), 50 mM sodium carbonate]
20   were added to each well. The enzymatic reaction was
allowed to proceed 1 hour and then the optical
density at 405 nm was determined in a TITERTEK
spectrophotometer available from Flow Laboratories,
Inglewood, CA.
25   E.   Cell Culture
        The ability of the receptor molecules of
this invention to immunoreact with EBNA produced in
cells was studied as described hereinabove using the
WI-L2, Raji, Daudi and BJAB cell lines. WI-L2 cells
30   (ATCC CRL 8155 W1L2-NS, American Type Culture
Collection, Bethesda, MD) are an EBV genome-positive
non-producer B-lymphoblast line, derived from a human
patient with hereditary spherocytic anemia. Levy, et
al., Cancer 22:517-524 (1968).

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Raji cells (ATCC CCL 86, Americal Type
Culture Collection, Bethesda, MD) are an EBV
genome-positive, EBNA procuding lymphoblast-like cell
line from a Burkitt lymphoma. Epstein, J. Nat.
5   Cancer Inst. 34: 231 (1965). Daudi cells (ATCC CCL
213, American Type Culture Collection, Bethesda, MD)
are also an EBNA producing cell line. BJAB cells are
a non-EBNA producing lymphocyte cell line available
at the Scripps Clinic and Research Foundation,
10   La Jolla, CA.

The above cell lines were cultured in RPMI
1640 medium [Moore, J. Am. Med. Assoc. 199:519-524
(1967); and Morton, In Vitro 6:89-100 (1970)]
supplemented with 2 mM L-glutamine and 10% fetal calf
15   serum.

F.   Whole Cell Extracts

Extracts of EBNA producing and non-producing
(control) cells were prepared to determine if
receptor molecules of this invention were useful for
20   diagnosing EBNA expression. Cells from cultures
described hereinabove were washed in
phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM
sodium phosphate, pH 7.4) containing 0.2 mM
phenylmethylsulfonyl fluoride, swollen for 5 minutes
25   in reticulocyte standard buffer (RSB; 10 mM NaCl, 10
mM Tris-HCl, pH 7.4, 1.5 mM MgCl$_2$, 0.2 mM
phenylmethylsulfonyl fluoride), and lysed by
sonication in 3-5 volumes of RBS adjusted to 0.2-0.35
molar (M) NaCl. After 30 minutes on ice, the
30   sonicate was centrifuged at 10,000xg for 15 minutes
to remove cellular debris.

G.   Immunoblotting Procedures

Cell extracts obtained above were assayed
for EBNA using human sera known to contain anti-EBNA
35   antibodies or exemplary receptor molecules of this

invention. The extracts were either concentrated by
precipitation with 2 volumes of ethanol at -20
degrees C. for about 18 hours, and were then
dissolved in sample buffer [SB; 10% glycerol, 2%
5   2-mercaptoethanol, 1% sodium dodecyl sulfate (SDS),
0.002% bromphenol blue, 40 mM Tris-HCl (pH 7.4)] or
diluted 1:6 in sample buffer for SDS-polyacrylamide
gel electrophoresis (SDS-PAGE). 7.5% Polyacrylamide
gels were cast and run according to the procedure of
10   Laemmli, Nature, 277:680-685 (1970), applying 50 to
200 micorgrams of total protein per lane.

         After electrophoresis, the protein bands
from the SDS polyacrylamide gels were transferred
electrophoretically to a solid support in the form of
15   nitrocellulose sheets (Schleicher and Schuell,
Detroit, MI) by the procedure of Towbin et al.,
Proc. Natl. Acad. Sci., U.S.A., 76:4350-5354 (1979).
This was accomplished using a Bio-Rad Trans-Blot
apparatus (Bio-Rad, Richmond, CA) at 70 volts for 2-3
20   hours in 12.5 mM Tris hydroxide, 96 mM glycine and
20% methanol.

         Following transfer, the nitrocellulose
filters or blots were saturated for one hour in
either 2% BSA (w/v) in PBS or 2% powdered milk (w/v)
25   in PBS to reduce non-specific binding. The blots
were than immunoreacted with 0.1 ml of either EBNA
positive human serum or rabbit anti-polypeptide
antibodies in 2 ml of PBS or 2% milk for 1 hour at 37
degrees C.

30   Anti-peptide antibodies bound to EBNA
protein were detected by reacting the blots with an
indicating means. In this instance, 20 ml of
^{125}I-labeled [200,000 counts per minute per
milliliter (cpm/ml), 10^6 counts per minute per
35   milligram (cpm/mg)] S. aureus protein A (Calbiochem,

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La Jolla, CA) were contacted with the immunoreaction product for 30 minutes at 37 degrees C. The blots were washed with PBS and exposed to Kodak XAR x-ray film overnight at -70 degrees C.

5 H. Immunizations

The receptor molecules of this invention include whole antibodies raised in mammals by immunizing them with inocula including a polypeptide and/or multimer as described hereinabove. Both

10 polypeptides and multimers may be used included in inocula alone or conjugated to a carrier protein such as keyhole limpet hemocyamin (KLH). However, polypeptides are preferably used as a conjugate and multimers are preferably used alone.

15 Rabbits were immunized with inocula containing 1.0 mg of conjugate in complete Freund's adjuvant (CFA), and boosted one month later with 1.0 mg of conjugate in incomplete Freund's adjuvant (IFA). Each immunization consisted of one

20 subcutaneous injection, on the back hip. Rabbits were bled 1 and 2 months subsequent to the boost.

Sera containing immunologically active antibodies were then produced from the bleeds by methods well known in the art. These antibodies

25 immunoreacted with one or more of the polypeptides of this invention, and an EBNA antigenic determinant. They may thus be used in a system to assay EBNA.

Individual inocula were prepared with CFA or IFA as follows: An amount of conjugate sufficient to

30 provide the desired amount of polypeptide per inoculation (e.g., 1 mg) was dissolved in PBS (at about 0.5 ml) at pH 7.2. Equal volumes of CFA or IFA were then mixed with the conjugate solutions to provide an inoculum containing conjugate, water and

35 adjuvant in which the water to oil ratio was 1:1.

The mixture was thereafter homogenized to provide the
inocula. The volume of an inoculum so prepared was
typically greater than 1 ml, and some of the
conjugate, PBS and adjuvant was lost during the
5   emulsification. Substantially all of the emulsion
that could be recovered was placed into a syringe,
and then was introduced into the rabbits as discussed
before. The amount of inoculum introduced into the
rabbits is believed to have been about 90 percent of
10   that present prior to the emulsification step.

        The above inocula stock solutions are
illustrative of the inocula of this invention. As
demonstrated herein, they may be used to produce
receptor molecules that immunoreact with EBNA.

15   I.   Immunofluorescense Procedures

        Another illustrative method for assaying
EBNA in a body sample uses receptor molecules of this
invention and a fluorochromatic indicating means to
detect the product of a receptor-EBNA immunoreaction.
20   In the present study, 2×10^4 WI-L2 cells,
grown as described above, were spread on a plain
microscope slide using a cytocentrifuge (CYTOSPIN,
Shandon Southern, Astmoor, Runcorn, Chesire,
England). After air drying for 5 minutes at 20°C,
25   the cells were fixed in acetone for 2 minutes, then
air dried for 2 minutes at 20°C. The slides were
stored at -20°C until used.

        The fixed WI-L2 cells were assayed for EBNA
using rabbit anti-polypeptide antibodies (receptors
30   of this invention) raised to polypeptides P27, P60,
P62 and P89. Fifty ul of each rabbit antiserum,
diluted 1:10 in VBS buffer (120 mM barbitol pH 7.3,
144 mM NaCl, 2.5 mM $MgCl_2$ and 0.75 mM$CaCl_2$) were
incubated (contacted and maintained in contact with
35   the fixed cells) at 20°C on a slide for a

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predetermined period of time (e.g. 30 minutes)
sufficient for the antibodies and EBNA to
immunoreact. A negative control slide was treated in
identical manner with normal rabbit serum.

5   During the above incubation, a portion of
the anti-polypeptide antibodies immunoreacted with
EBNA present in the fixed WI-L2 cells. Unbound
antibodies were removed by washing with VBS, leaving
only the EBNA-receptor immunoreaction product on the
10   slide.

Anti-polypeptide antibodies bound to EBNA
were detected by first incubating 50 ul of guinea pig
complement (Tago, Burlingame, CA) diluted 1:10 in VBS
on each slide for a period of time (30 minutes)
15   sufficient for the complement to bind to the
receptors. The slides were then washed with VBS to
remove any complement not bound to the rabbit
anti-polypeptide IgG.

Fluorescein labeled goat anti-guinea pig
20   C_3 (labeled anti-complement antibodies, Cappel
Laboratories, Cochranville, PA) was used to detect
the antigen-antibody-complement complexes. Fifty ul
of the indicating antiserum, diluted 1:20 in VBS,
were incubated as above on each slide for 30 minutes
25   at 20°C. Unbound goat anti-guinea pig C_3 was
washed off the slide with VBS. Immunoreaction
products were then visualized by fluorescent
microscopy.

J.   Circular Dichroism Spectroscopy
30   The conformational properties of the
polypeptides were investigated to elucidate any
secondary structure that might be necessary for
polypeptide immunoreaction with human anti-EBNA
antibodies. The polypeptides were dissolved at a
35   concentration of 1 mg/ml in phosphate-buffered saline

(PBS). Spectra were taken using 1 ml samples in a Cary 61 spectropolarimeter (Cary Instruments, Applied Physics Corp., Monrovia, CA) interfaced and automated with a Digital Equipment Corporation 11/02 computer

5   (Digital Equipment Corporation, Maynard, MA). The average of 10 successive scans for each polypeptide was plotted as shown in Figure 1.

        The foregoing is intended as illustrative of the present invention but not limiting. Numerous

10   variations and modifications may be effected without departing from the true spirit and scope of the novel concepts of the invention. It is to be understood that no limitation with respect to the specific polypeptides, antibodies, their compositions and uses

15   illustrated herein is intended or should be inferred.

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What is Claimed Is:

      1.   A synthetic, random copolymer
polypeptide containing about 6 to about 40 amino acid
residues and including the five amino acid residue
sequence, written from left to right and in the
direction of the amino-terminus to carboxy-terminus,
represented by the formula:

$$-Gly-R^1-Gly-R^2-Gly-$$

      wherein:

      R^1 and R^2 are amino acid residues that
individually are the same or different and are
selected from the group consisting of Ala, Asn, Arg,
Gly, Leu, Pro, Ser and Thr, with the provision that
both R^1 and R^2 are not Gly;

      said polypeptide containing at least 25 mole
percent glycine residues; and

      said polypeptide being capable of inducing
the production of antibodies that immunoreact with
EBNA when linked to a carrier and introduced in an
effective amount into a mammalian host.

      2.   The synthetic polypeptide according to
claim 1 wherein the included amino acid residue
sequence is selected from the group consisting of:

      (i)   -Gly-Ala-Gly-Ala-Gly-;

      (ii)   -Gly-Ala-Gly-Gly-Gly-;

      (iii)   -Gly-Gly-Gly-Ala-Gly-;

      (iv)   -Gly-Arg-Gly-Arg-Gly-;

      (v)   -Gly-Asn-Gly-Leu-Gly-; and

      (vi)   -Gly-Ser-Gly-Ser-Gly-.

      3.   The synthetic polypeptide according to
claim 1 wherein said polypeptide:

      (a)   contains at least 50 mole percent
glycine residues;

      (b)   includes an additional six amino acid
residue sequence that overlaps with said five residue

sequence, said six residue sequence, written from
left to right and in the direction of amino-terminus
to carboxy-terminus, being represented by the formula:

<div align="center">-Gly-Ala-Gly-Gly-Ala-Gly-; and</div>

5   (c) is capable of immunoreacting with human
antibodies induced by EBNA.

4.   The synthetic polypeptide according to
claim 3 wherein the amino acid residue sequence of
said polypeptide includes a sequence, written from
10   left to right and in the direction of amino-terminus
to carboxy-terminus, selected from the group
consisting of:

(i)   -Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-
Gly-Ala-Gly-Gly-Gly-Gly-Arg-;

15   (ii)   -Lys-Gly-Thr-His-Gly-Gly-Thr-Gly-Ala-
Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-
Ala-Gly-;

(iii)   -Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-
Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-
20   Gly-Ala-Gly-;

(iv)   -Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-
Gly-Ala-Gly-Gly-Gly-Ala-Gly-;

(v)   -Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-
Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-
25   Ala-Gly-Ala-Gly-;

(vi)   -Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-
Gly-Ala-Gly-Gly-Ala-Gly-Gly-;

(vii)   -Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
Gly-Gly-Gly-Ala-Gly-Ala-Gly-;

30   (viii)   -Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-
Ala-Gly-Ala-Gly-, the
pharmaceutically acceptable salts
thereof, and antigenically related
variants thereof.

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5.   The synthetic polypeptide of claim 3
wherein said amino acid residue sequence contains
about 15 to about 20 amino acid residues.

6.   A synthetic multimer containing a
plurality of joined synthetic random copolymer
polypeptides of claim 1 as repeating units.

7.   The multimer according to claim 6,
wherein said polypeptide repeating units are joined
together by a bond selected from the group consisting
of an amide and a disulfide.

8.   A synthetic polypeptide inoculum
suitable for inducing antibodies that immunoreact
with EBNA that comprises a synthetic random copolymer
polypeptide of claim 1 dissolved or dispersed in an
effective amount in a pharmaceutically acceptable
diluent,

said inoculum when introduced in an
effective amount into a mammalian host being capable
of inducing the production of antibodies that
immunoreact with EBNA.

9.   The synthetic polypeptide inoculum of
claim 8 wherein said polypeptide is bound to a
carrier.

10.   A synthetic polypeptide inoculum
according to claim 8 in unit dosage form wherein the
polypeptide is present in an amount of about 10
micrograms to about 500 miligrams per dose.

11.   A receptor molecule containing an
antibody combining site raised to a synthetic
immunogen, the synthetic immunogen comprising a
synthetic, random copolymer polypeptide of claim 1.

12.   A diagnostic system in kit form for
assaying for the presence of EBNA comprising:

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         a)   receptor molecules raised to a
synthetic random copolymer polypeptide of claim 1;
and

         b)   indicating means for signaling the

5   immunoreaction of the receptor with EBNA.

         13.   A method of assaying for anti-EBNA
antibodies in a body sample comprising the steps of:

         a)   providing a body sample to be assayed;

         b)   admixing said body sample with a

10   synthetic, random copolymer polypeptide of claim 3;

         (c)   maintaining said admixture for a
predetermined time sufficient for anti-EBNA
antibodies present in said sample to immunoreact with
said polypeptide; and

15   (d)   determining the presence of said
immunoreaction.

         14.   The method of claim 13 including the
further step of affixing said polypeptide to a solid
support prior to said admixing.

20   15.   A diagnostic system in kit form for
assaying for the presence of antibodies to EBNA in a
body component comprising in separate packages:

         a)   a synthetic, random copolymer
polypeptide of claim 3; and

25   b)   an indicating means for signaling the
immunoreaction of said polypeptide with antibodies to
EBNA.

         16.   The diagnostic system of claim 15
wherein said synthetic polypeptide is affixed to a

30   solid matrix to form a solid support.

         17.   The diagnostic system of claim 16
wherein said indicating means is a labeled antibody
capable of immunoreacting with human anti-EBNA
antibodies.

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18. A preparation for passive immunization against B lymphocytes expressing EBNA on their cell surfaces comprising an effective amount of antibodies raised to a synthetic polypeptide of claim 1.

19. A method of assaying for the presence of EBNA in a body sample comprising the steps of:

a) providing a body sample to be assayed;

b) admixing receptor molecules containing an antibody combining site reaised to a synthetic, random copolymer polypeptide of claim 1;

c) maintaining said admixture for a predetermined time sufficient for said receptor molecules to immunoreact with EBNA present in said body sample; and

d) measuring the amount of said immunoreaction.

20. An immunoreaction product comprising a human anti-EBNA antibody immunologically bound to a synthetic, random copolymer polypeptide of claim 3.

FIG. 1
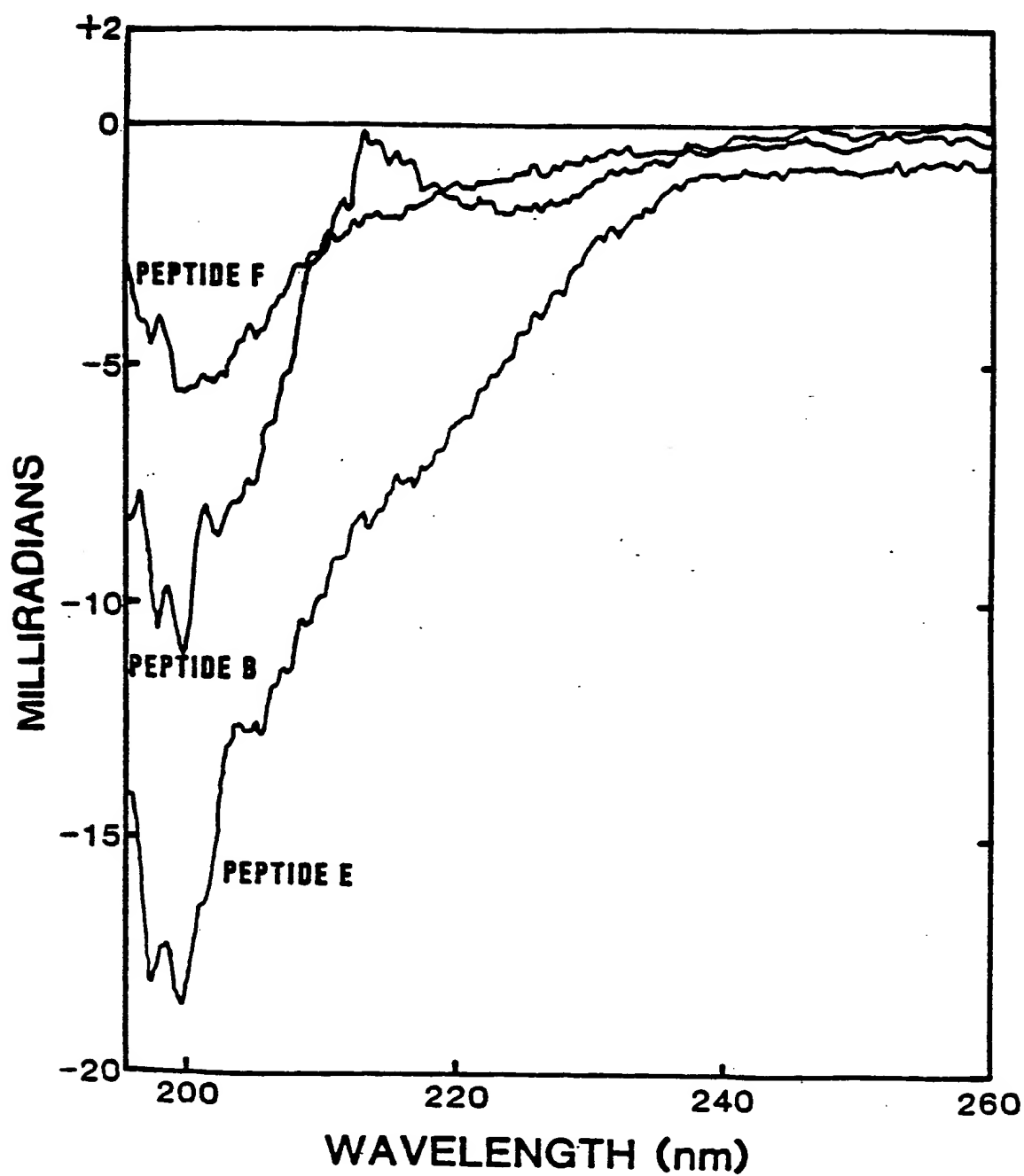
FIG. 2

PEPTIDE

ANTISERA EBNA C C B B B B

INHIBITOR — — C — B — C
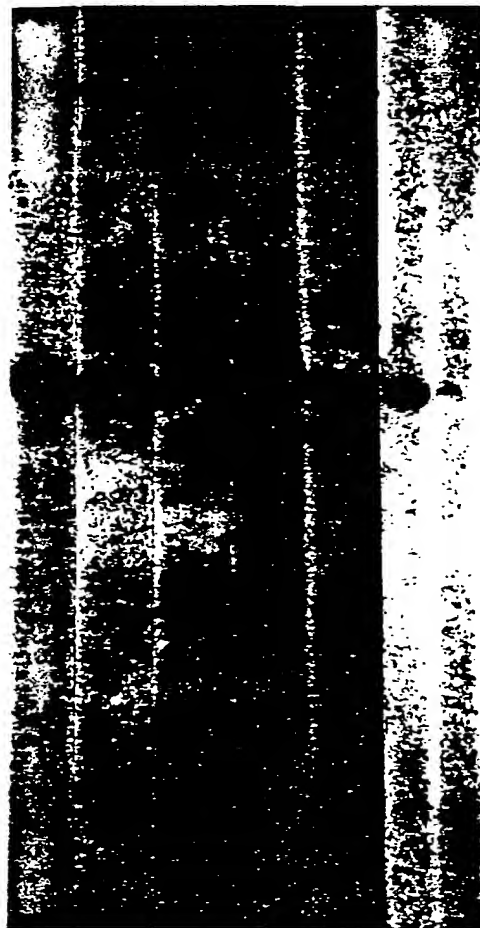
A B C D E F G

FIG. 3
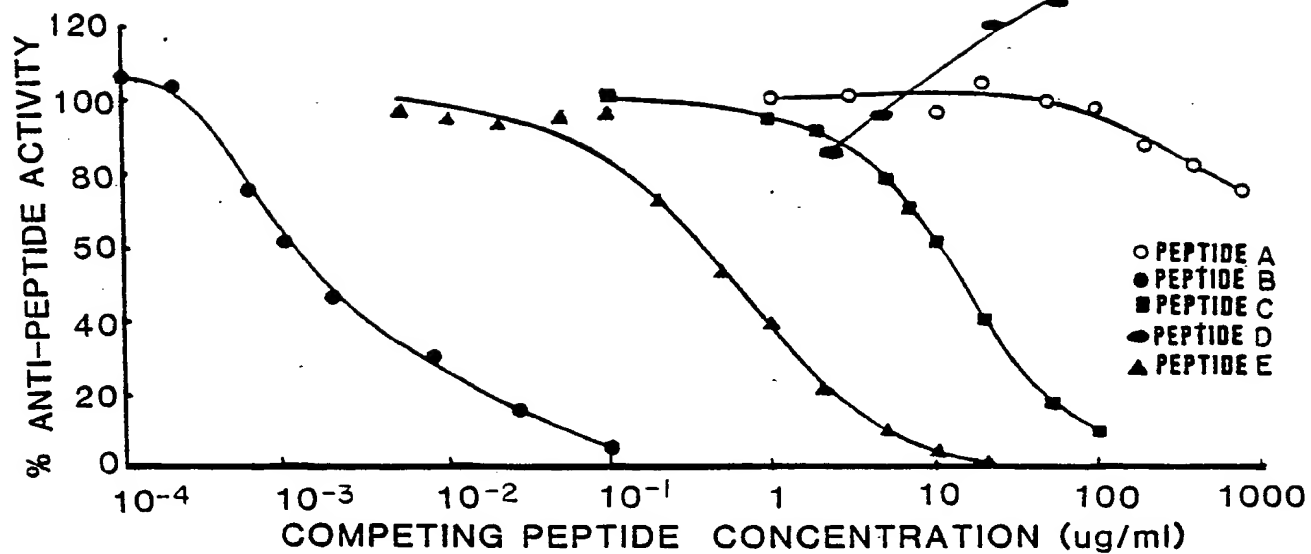
FIG. 4
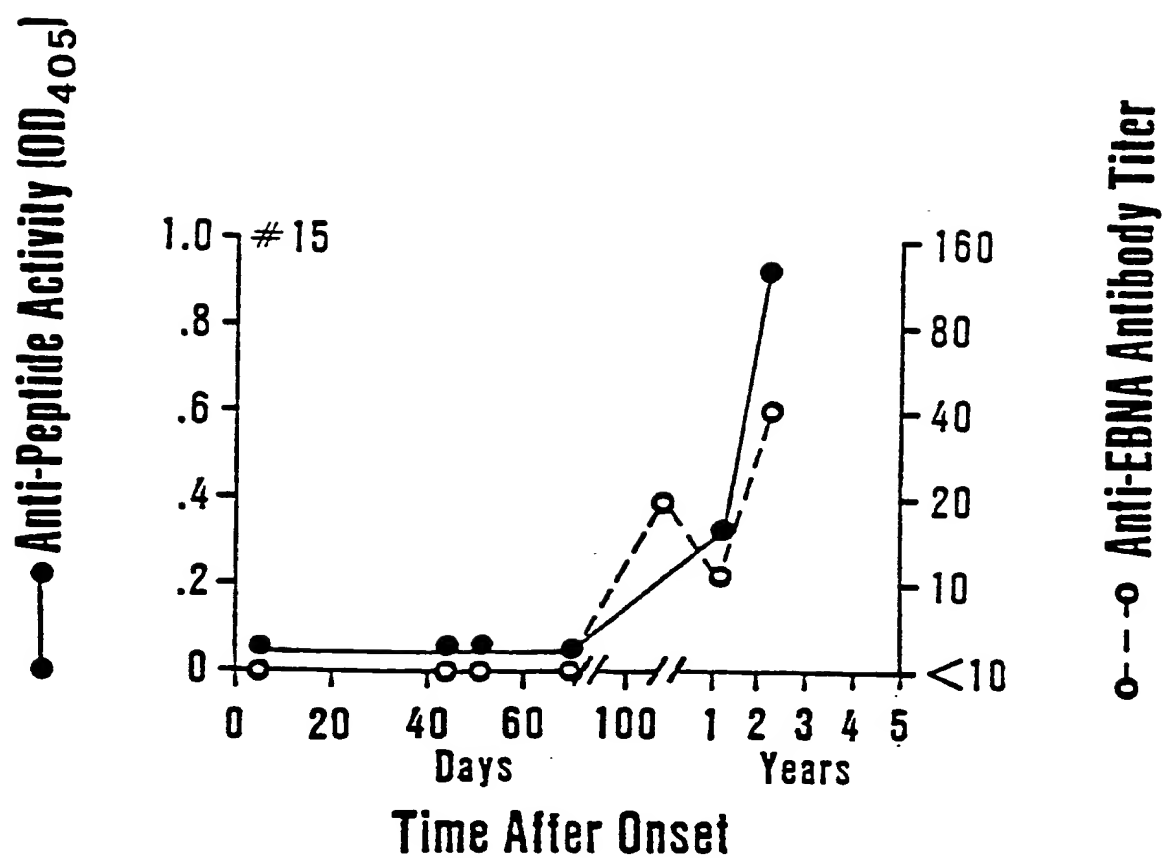
FIG. 5
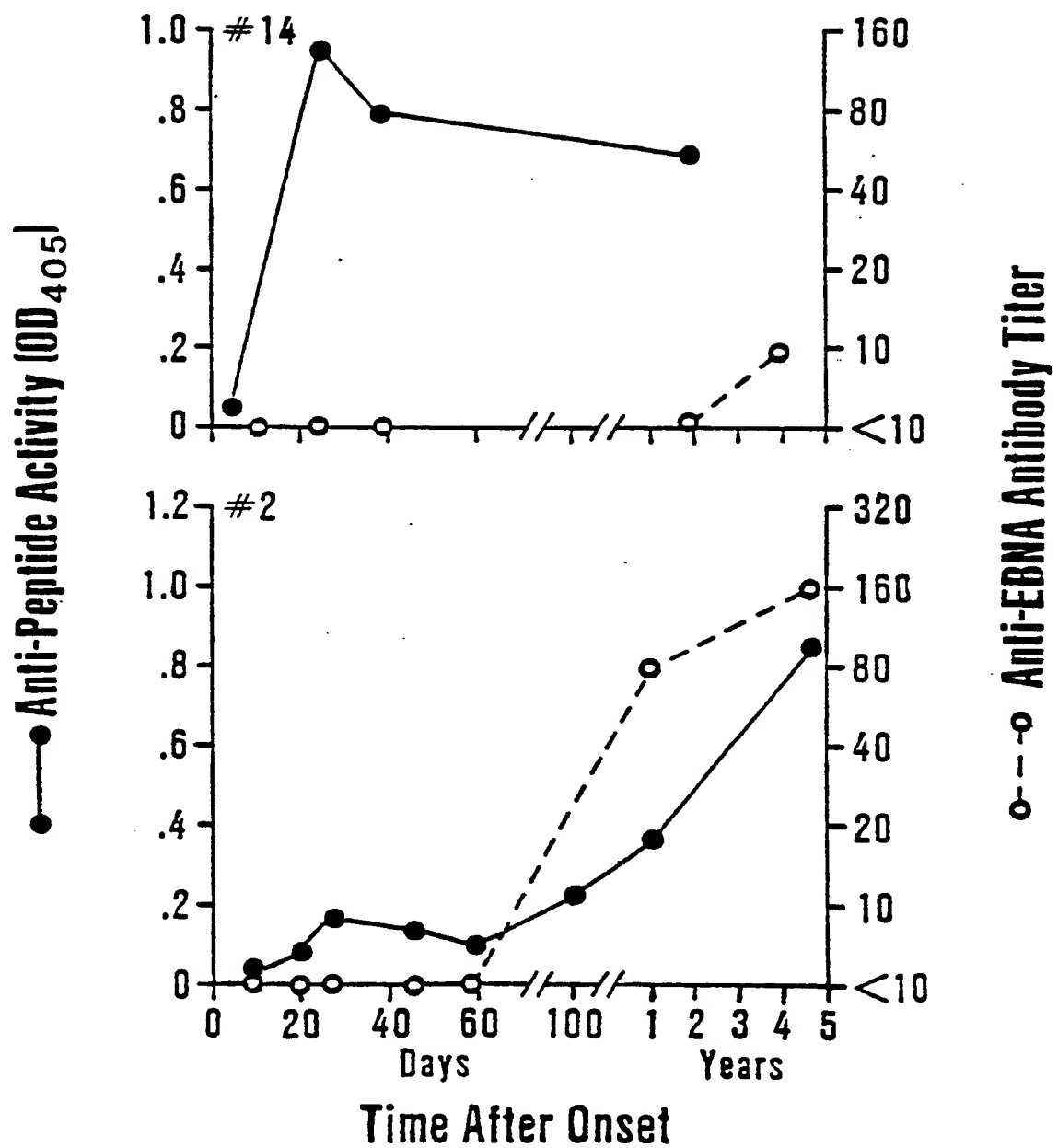
INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01484

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL^4 C07K 7/00,17/02; G01N 33/531; U.S.CL. 260/112.5R;
424/85,86; 435/5,7; 436/518,547,808,812,823

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	260/112.5R; 424/85,86; 435/5,7; 436/518,547, 436/808,812,823

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS, 1977-1984 UNDER "VIRUS, EPSTEIN-BARR"

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US,A, 3,959,456 Published 25 May 1976, Zichis	
A	US,A, 4,224,306 Published 23 September 1980, Zichis	
Y	US,A, 4,407,965 Published 04 October 1983, Yanaihara	1-20
Y	US,A, 4,423,034 Published 27 December 1983, Nakagawa	1-20
A,P	US,A, 4,474,886 Published 02 October 1984, Willard	
Y	N,J.Immunol.Methods, Vol. 67, No. 1, issued 1984, J. Luka et al 'A Sensitive Enzyme-Linked Assay (ELISA) Against the Major EBV-Associated Antigens', pages 145-156	12-17

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10 October 1985	17 OCT 1985
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/USA	Sidney Marantz

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	N,Proc.Natl.Acad.Sci. USA, Vol. 80, No. 18, issued September 1983, K. Hennessy et al., 'One of two Epstein-Barr virus nuclear antigens contains a glycinc-alanine copolymer domain', pages 5665-5669	1-7
X	N,Proc.Natl.Acad.Sci, USA, Vol. 81, No. 15, issued August 1984, J. Dillner et al., 'Antibodies against a synthetic peptide identify the Epstein-Barr virus-determined nuclear antigen', pages 4652-4656.	1-20

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